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CANCER RESEARCH

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Studies with Radioactive Carbon-Labeled Acetate on Cholesterol Metabolism in Rats Fed p-Dimethylaminoazobenzene*

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Information concerning the cholesterol metabolism of tumor-bearing animals is indeed small and also somewhat conflicting, in part due to lack of comparisons with homologous normal tissues. Bennet (1) found that as the age of the Flexner-Jobling tumors increases in rats the percentage of cholesterol likewise increases. These findings were confirmed by Iwano (2) and by Uramoto (3), the latter finding an increase in both free and combined cholesterol. The cholesterol content of the central portion of various tumors has been found to be higher than that of the periphery (2, 4). Bronstein and Volkensohn (4) found no change in the cholesterol content of the periphery of Jensen rat sarcoma which had been irradiated with X-ray *in vivo;* they also found growth of sarcoma cells *in vitro* produced no change in the cholesterol content of the medium. More cholesterol has been found in various human malignant tumors than in benign ones, both by histological (5) and by chemical (6) analysis. Also, in transplantable-carcinoma-bearing mice an increase in the cholesterol esters of the whole organism has been reported (7). Finally, Kahn (8) has reported a lowered content of cholesterol and its esters in blood serum of patients bearing malignant tumors.

That acetate can be converted into cholesterol was first indicated by the work of Sonderhoff and Thomas (9) in their study on oxygenated yeast with deuterio-acetate as the sole nutrient and the finding of deuterium in the unsaponifiable fraction and by the work of Rittenberg and Bloch (10) in their study of deuterio-acetate fed animals and the isolation of deuterium-labeled cholesterol. More recently a review on acetate metabolism by Bloch (11) has appeared.

The work herein reported was carried out in an attempt to further the knowledge of cholesterol metabolism in tumors, and in a tumor that could be compared with its homologous normal tissue.

METHODS

Animals, feeding, injections, and autopsy.—Long Evans strain stock rats from a highly-inbred colony, were placed on the experimental diets at the age of 8 weeks. Two diets were used, each consisting of a modified stock diet of 10.6 per cent protein, 16.2 per cent fat and 61.7 per cent carbohydrate, but the butter yellow diet contained, in addition, 0.06 per cent p-dimethylaminoazobenzene. The diet contained sterols which formed 7.9 mg. digitonides per gm. The animals were given *ad libitum* amounts of the diet for a period of 8 months at which time subcutaneous injections of a $CH_3C^{14}OONa$ solution were begun for a period of 5 days, injections being made every 12 hours for a total of 10 injections. The doses given were determined on a weight basis, a total of 3.97×10^{-2} μc, or 4.96×10^{3} counts per minute, per gram of rat tissue being injected. The solution was prepared by weighing out the $CH_3C^{14}OONa$ (6.7 μc/mg.) on a microbalance and carefully diluting with physiological saline to make a 0.01181 per cent solution. The animals were killed by ether approximately 4 hours after the last injection, the individual organs removed, frozen rapidly, and stored at −18° C. until they could be worked up. Small pieces of tissue were removed for histological examination, these being taken from the main tumor area in the case of

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tumor formation or from the posterior edge of the normal-appearing livers. The blood samples were pooled at autopsy.

Isolation of the cholesterol.—Individual organs were worked up, including whole hepatomatous livers. The method used was one based on that of Schoenheimer and coworkers (10, 12), in which the tissues are dissolved and saponified by 2 hours of refluxing with 7 per cent alcoholic sodium hydroxide. The non-saponifiable fraction is recovered by ether extraction and washed with water until neutral. After the ether was evaporated off, the dry residue was taken up in hot 95 per cent ethanol and a calculated 10 per cent excess of digitonin added as a 1 per cent solution in 90 per cent ethanol. After standing overnight the digitonides were filtered onto disks of filter paper which had been previously washed, dried and weighed.

Determination of the radioactivity.—The digitonides were weighed after drying and counted as digitonides with a thin mica window, bell-type Geiger-Müller counter to a statistical error of less than 3 per cent.[1] Corrections for self-absorption of the beta-rays were made by using a curve prepared from C^{14}-labeled protein; this is permissible since the protein is composed of atoms of atomic weight similar to those found in cholesterol-digitonide (13–16). Since $\frac{1}{4}$ of the cholesterol-digitonide is cholesterol (17) calculations were made on this basis. As a check on this procedure some of the digitonide samples were split by the pyridine method (18) and recrystallized from 85 per cent ethanol.

Feces.—The feces were collected and worked up in a manner similar to that for the tissues. Calculations were made as though the sterols present were cholesterol.

Urine.—The urine from the female rats which were on the butter yellow diet was collected, stored under toluene in the cold, then hydrolyzed and extracted simultaneously by the method of Dingemanse, Borchardt and Laquer (19). After distilling off the carbon tetrachloride, the residue was taken up in benzene; this benzene extract was evaporated to dryness on an aluminum disk and counted under the Geiger-Müller counter.

RESULTS

Animal groups.—The results allow division of the animals into three groups: a control group (I) of four animals on the diet containing no butter yellow, a butter yellow tumorous group (II)[2] of four animals on the diet containing the butter yellow and which developed tumorous livers, and a butter yellow non-tumorous group (III) on the diet containing the butter yellow, but which developed no tumorous livers. One other animal which was on the butter yellow diet developed a lung tumor; it was worked up the same way as the other animals. The average weight in grams during the period of injections for group I was 290; for II, 233; and for III, 235, for the animal with a lung tumor, 144. There were two females in group I, three in group II, three in group III; the animal with a lung tumor was a female; the rest were males.

Pathology.—Grossly, the livers of the animals in group II were firmer than normal, showing a slight hob-nail appearance, several cysts, and nodules which were firm and cream colored. The sizes of the nodules in each of the 4 livers were as follows: (a) a 10 mm. nodule in upper right lobe and a 12 mm. nodule in the lower right lobe appearing some what cystic; (b) two 8 mm. nodules in lower right lobe; (c) a 20 mm. nodule, cystic, in lower right lobe; (d) a 25 mm. nodule in lower right lobe, a 5 mm. nodule in the lower left lobe, and numerous smaller (1 to 2 mm.) nodules throughout the entire liver. Necrosis was not pronounced in any of the animals. In animal (d) there was also a firm nodule, 5 mm. in diameter, in the mesentery, apparently either an implant or a metastasis. The animal with the lung tumor had a large cystic mass, 25 mm. in diameter, on the right side, apparently originating in the lower lobe of the lung. The left side showed compensatory hypertrophy, and there was an empyema pocket, 7 mm. in diameter, just above the diaphragm.

Microscopically, the liver tumors were typical of those described in the literature (cf. Edwards and White (20), and Opie (21)), the tumorous animals all having hepatoma type II (20). Two of the animals had, in addition, adenocarcinoma and all showed some cirrhosis and fatty degeneration. The nodule from the mesentery consisted mainly of fibrous connective tissue with a few lymphopoietic centers and with a few anaplastic cells, some of which resembled liver cells. The livers of groups I and III were normal except for some cirrhosis and fatty degeneration. The lung tumor appeared to be a bronchogenic carcinoma.

Percentage of cholesterol, radioactivity and organ size.—The results are summarized in Tables 1, 2, and 3. The percentages are highest for adrenals and brain and lowest for the whole blood. The per cent of cholesterol in tumorous livers is higher than that in the control livers[3]. The conditions are exactly reversed in the spleens. It can be seen that the highest specific activity is found in the cholesterol derived from the stomach and intestines and the

[1] Autoscaler counter with mica window of 1 to 2 mgm. per cm^2.

[2] Tumorous is used throughout this paper to mean hepatoma, type II (20); two of the animals had in addition to the hepatoma, adenocarcinoma.

[3] The difference of the means is 2.4 times the probable error (only 1 chance in 15.6 that a random error exceeds the value). Since small groups were used the statistical treatment utilizes a modification of the Gaussian theory of errors by Jeffreys (22) which purports to yield precision measures that are more reliable.

lowest in that from the brain. Although the specific activity of the cholesterol derived from the tumorous livers is higher than that of the cholesterol derived from the control livers, the difference is not significant. There are some definite differences in the ratio of the weight of the animal to the weight of the organ in groups I and II. In group II there is a definite decrease in the ratio from the control values in liver, testes and spleen.

Feces.—Two groups make up the data on the feces: group I as above for the controls, and a group of 6 females on the butter yellow diet (3 with liver tumors, 1 with a lung tumor, and 2 with no tumors). On a dry weight basis the percentage of sterol (mainly coprosterol with some dihydrocholesterol) in the controls is 0.37 while in the butter yellow group it is 0.42. The specific activity, calculated as cholesterol is 6.12 counts per minute per mg. of cholesterol for the control group and 6.05 for the butter yellow group.

Urine.—The residue from the benzene extract showed a moderately high radioactivity.

Splitting of the digitonides.—It was observed that counting the samples as the digitonides results in specific activities about 15 per cent lower than counting the pure cholesterol.

TABLE 1

CHOLESTEROL TURNOVER OF TISSUES OF CONTROL RATS

Organ	Av. animal wt. gm.	Av. organ wt. gm.	Cholesterol per cent wet wt.	Specific activity of cholesterol cts./min./mg.	Cts./min. gm. of organ
Intestines and Stomach	290 ±66	9.00±0.71	0.24±0.032	22.7±2.42	54.4
Adrenals	"	0.049	6.20	19.6	1218.0
Blood (whole)	"		0.11	18.4	
Spleen	"	0.73±0.14	0.59±0.169	17.6±5.08	103.6
Liver	"	9.57±1.17	0.33±0.030	17.5±3.08	57.7
Lungs	"	1.38±0.31	0.56±0.111	17.3±5.23	97.2
Kidneys	"	1.52±0.34	0.45±0.120	8.9±1.52	39.8
Testes	335.0	1.21	0.43	6.6	28.6
Brain	290 ±66	1.92±0.20	2.46±0.087	0.48±0.16	11.7

Dose of $CH_3C^{14}OONa$ per gm. of rat tissue in cts. per min.: 4.96×10^3.
± values are expressed as standard deviation from the mean.

TABLE 2

CHOLESTEROL TURNOVER OF TISSUES OF BUTTER YELLOW TUMOROUS RATS

Butter Yellow Tumorous: Organ	Av. animal wt. gm.	Av. organ wt. gm.	Cholesterol per cent wet wt.	Specific activity of cholesterol cts./min./mg.	Cts./min. gm. of organ
Intestines and stomach	233 ±52	9.20±0.49	0.23±0.037	22.3 ±2.77	51.4
Adrenals†	182.0	0.031	7.28	20.2	1472.0
Blood (whole)*	203.0		0.13	19.7	
Spleen	233 ±52	1.52±0.73	0.35±0.109	20.2 ±3.24	70.7
Liver	"	15.55±7.17	0.40±0.046	19.6 ±3.50	78.3
Lungs	"	1.17±0.45	0.45±0.103	19.6 ±2.28	88.2
Kidneys	"	1.24±0.66	0.35±0.091	12.3 ±1.63	43.1
Testes	321.0	3.47	0.18	6.6	7.7
Brain	233 ±52	1.64±0.25	2.48±0.525	0.72±0.08	17.9

Dose of $CH_3C^{14}OONa$ per gm. of rat tissue in cts. per min.: 4.96×10^3.
* Females only.
† All butter yellow females (6 animals); ± values are expressed as standard deviation from the mean.

TABLE 3

CHOLESTEROL TURNOVER OF TISSUES OF BUTTER YELLOW NON-TUMOROUS RATS

Organ	Av. animal wt. gm.	Av. organ wt. gm.	Cholesterol per cent wet wt.	Specific activity of cholesterol cts./min./mg.	Cts./min. gm. of organ
Intestines and stomach	235 ±84	8.95±1.67	0.24±0.017	22.3 ± 2.46	53.5
Blood (whole)*	174.0		0.17	22.6	
Spleen	235 ±84	1.07±0.29	0.24±0.116	14.2 ±12.28	34.6
Liver	"	9.52±1.14	0.37±0.050	19.7 ± 8.88	72.7
Lungs	"	1.00±0.37	0.52±0.008	18.5 ± 8.22	96.0
Kidneys	"	1.33±0.58	0.32±0.125	10.6 ± 2.11	34.6
Testes	321.0	2.78	0.31	3.9	12.2
Brain	235 ±84	1.65±0.32	2.42±0.213	0.55± 0.02	13.3

Dose of $CH_3C^{14}OONa$ per gm. of rat tissue in cts. per min.: 4.96×10^3.
± values are expressed as standard deviation from the mean.
* Females only.

DISCUSSION

One indication of the ability of an organ, per unit weight, to form cholesterol is a comparison of values derived from the expression:

$$\frac{\text{counts/min. obtained as cholesterol/gm. organ tissue}}{\text{counts/min. given as acetate/gm. rat tissue}} \times 100$$

which might be termed the conversion efficiency. The values are recorded in Table 4. On this basis, it is at once obvious that the adrenal gland is the most efficient producer of cholesterol. The values, however, must be interpreted with caution because of two factors: the percentage of inert cholesterol diluting the radioactive species and the rate of acetate production by the organ concerned. Thus, the low specific activity of the brain cholesterol might be explained, at least in part, by the dilution with the large amount of cholesterol present. Indeed, Waelsch *et al.* (23) found that the rate of deposition of lipids in the brain (and the data indicate the lipids are probably synthesized in the brain itself from fifteenth to nineteenth day) was high immediately after birth, but decreased rapidly up to the thirtieth day such that little seems to be deposited after that time. Still another interpretation presents itself: the possibility that the blood-brain barrier (24) is effective here[4]. In the case of the adrenals, however, since the percentage cholesterol is higher than in brain and the specific activity is high, it seems likely the production of cholesterol by this organ is very great. Data on acetate production for individual organs are not available.

In view of the production of hormones with structures similar to that of cholesterol by the adrenal glands, the efficiency of this gland in cholesterol production is of great interest (cf., Long (28)). Srere *et al.* (29) in a recent experiment with adrenal cortex slices, utilizing doubly labeled sodium acetate with C^{14} in the incubation medium, found that this tissue could convert acetate into cholesterol. Because of the similar structure of the adrenocortical hormones and cholesterol, and because Bloch (30) has shown that deuterium-labeled cholesterol can be converted into deuterium-labeled pregnanediol in the pregnant woman, the possibility of finding labeled 17-ketosteroids in the urine of animals given labeled acetate appears likely. This view is supported by the finding of deuterium in both the nucleus and the side-chain of deuterium-labeled cholesterol.[5] The pooled urines were therefore partially worked up with possible 17-ketosteroid isolation in mind, though the small amount expected presented difficulty. Although a moderately high activity was found in the residue from the benzene extract, it was not believed feasible to isolate the ketonic fraction with Girard's T reagent in view of the relatively low levels of radioactivity administered. Subsequent work is planned along these lines.

TABLE 4

EFFICIENCY OF CONVERSION OF ACETATE TO CHOLESTEROL BY DIFFERENT TISSUES

	Cts./min. obtained as cholesterol per gm. organ tissue / Cts./min. given as acetate per gm. rat tissue ×100		Cts./min. per mole cholesterol / Cts./min. per mole acetate ×100		Cts./min. per mole cholesterol per gm. organ tissue / Cts./min. per mole acetate per gm. rat tissue ×100	
Organ	Controls	Tumorous	Controls	Tumorous	Controls	Tumorous
Intestines and stomach	1.1	1.0	1.27×10^{-2}	1.25×10^{-2}	0.40	0.32
Adrenals*	24.6	29.6	1.10×10^{-2}	1.13×10^{-2}	65.1	66.5
Spleen	2.1	1.4	9.85×10^{-3}	1.13×10^{-2}	3.91	1.66
Liver	1.2	1.6	9.81×10^{-3}	1.11×10^{-2}	0.30	0.16
Lungs	2.0	1.8	9.73×10^{-3}	1.10×10^{-2}	2.04	2.21
Kidneys	0.8	0.9	4.96×10^{-3}	6.89×10^{-3}	0.91	1.33
Testes	0.6	0.2	3.72×10^{-3}	3.67×10^{-3}	1.03	0.34
Brain	0.2	0.4	2.67×10^{-4}	4.05×10^{-4}	0.04	0.06

* In the tumorous group, females only.

[4] When labeled glycine, methionine or tyrosine are given subcutaneously and the trichloracetic acid precipitable fraction from brain is tested for radioactivity, the specific activity is much lower than that of corresponding fractions from other organs (cf., 25, 26, 27). If, however, labeled glycine is injected into the cerebrospinal fluid directly the brain fraction is higher than those from other organs (unpublished experiments from this laboratory).

[5] Determined by Bloch and Rittenberg (31) by converting the cholesterol to cholesteryl chloride and subjecting the latter to thermal degradation; the deuterium concentrations found in the isooctane-isooctene mixture and in the hydrocarbon $C_{19}H_{30}$ were in good agreement with that in the cholesteryl chlorides.

The finding of C^{14} in the coprosterol-dihydrocholesterol digitonides from the feces brings up the question of the source of the C^{14}. Does it arise by the reaction postulated below:

$$\begin{array}{ccccc} & & \text{Oxid.} & & \text{Red'n.} \\ \text{Acetate} \rightarrow & \text{Cholesterol} & \rightleftarrows & \text{Cholestenone} & \rightleftarrows \quad \text{Coprostanone} \\ & & & \uparrow\downarrow \text{Red'n.} & \uparrow\downarrow \text{Red'n.} \\ & & & \text{Dihydrocholesterol} & \text{Coprosterol (32, 33),} \end{array}$$

or can acetate form intermediates other than cholesterol in the reactions directly? Bloch and coworkers, found *in vivo* transformation of deuteriocholesterol into deuteriocholic acid (34) and into deuterium-labeled pregnanediol (30), and because it was found that a minimum of two-thirds of these steroids can arise by degradation of cholesterol, it was concluded (11) that it is unlikely that animal steroids other than cholesterol can be formed by total synthesis from small units by a pathway in which cholesterol is not an intermediate. According to the work of Schoenheimer and coworkers (32, 35, 36) and of Rosenheim and Webster (33), the above scheme is the most probable one; dihydrocholesterol being formed in the tissues (35) by a reduction of cholesterone and then excreted through the intestinal wall (36), where a large part of it is converted into coprosterol, presumably by the intestinal flora. Further studies with labeled steroids, especially cholestenone would be of great value in testing the above pathway.

The fact that the specific activity of the cholesterol is higher when it is counted as cholesterol than as the digitonide can be explained on the basis that the correction curve used for self-absorption was constructed from data on a material other than cholesterol digitonide itself. Srere *et al.* (29), in the experiment mentioned above, found that the specific activity of the cholesterol in the digitonides was higher than when counted as pure cholesterol or as cholesteryl acetate, but the number of counts added per incubating flask was greater than the number given to the whole animal in the work here reported, making it more difficult to remove contaminating acetate.

The animal with the lung tumor showed the following results: weight, 144 gms.; average weight of organ, per cent of cholesterol, specific activity of the cholesterol in counts per minuts per mgm., respectively: intestines and stomach: 6.17 gms., 0.30 per cent, 18.4; spleen: 0.41 gms., 0.44 per cent, 13.4; liver: 4.44 gms., 0.35 per cent, 21.3; lungs: 8.30 gms., 0.43 per cent, 9.1; kidneys: 0.86 gms., 0.41 per cent, 15.0; brain: 1.30 gms., 2.93 per cent, 0.56.

That no significant differences between the specific activities of liver cholesterol of groups I and II were found might be explained on the basis that total liver was taken. If the tumor tissue had been dissected out and had been run separately, differences might have been found. Tissue slice would be important in this respect, as would also degradation of the labeled cholesterol to find the location of the labeled atoms, thus possibly giving clues as to the intermediates in cholesterol synthesis.

SUMMARY

1. Radioactive carboxyl-labeled acetate was injected into normal rats and into rats bearing butter yellow-induced hepatomata and the labeled cholesterol was isolated from various individual whole organs by the digitonide method.

2. No significant differences were found in the specific activities of the liver cholesterol in the two groups.

3. A higher percentage of cholesterol was found in the livers of the tumor-bearing animals than in those of the control rats.

4. The ability of the adrenal tissue per unit weight to make cholesterol is much greater than that of any other tissue studied.

5. The coprosterol and dihydrocholesterol digitonides isolated from feces were also radioactive. A discussion of the probable metabolic pathway is presented.

6. The occurrence of a lung tumor in one of the rats which received the butter yellow diet and its corresponding cholesterol study is reported.

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Cancer Innervation

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The problem as to whether or not malignant tumors are innervated has been a controversial one since the question first arose in the eighteenth century. Approximately a hundred papers have been recorded on the subject of nerves in tumors, but the common conclusion (with only a few exceptions) is that nerve fibers can be found in malignant growths. However, opinions diverge as to their meaning.

Young (18), Itchikawa and Takahata (7), Martynow (9), Athias and Dias (1), Oertel (11, 12, 13, 14), and others interpreted these nerve fibers as an integral part of the cancerous growth. Herzog (6), Ludford (8), Ryrie (15), Willis (17), and others concluded that the growth of a malignant tumor is independent of any specific nervous organization, and the presence of nerves in cancer is due, either to the persistence of pre-existing nerves, or to nerves becoming included within the cancer owing to the invasive character of its growth. A few authors suggested the possibility of a vasomotor function for some of the nerve fibers. More recently, Duncan and Bellegie (3) reported the penetration of an experimental sarcoma by nerve fibers from the spinal cord. For a more complete summary of the literature on this subject reference may be made to the papers of Athias and Dias (1), and that of Tavares (16).

All of these reports were based upon morphological studies, and the conclusions drawn were naturally subject to the vagaries of the particular staining procedure employed and the individual interpretation of the investigator. In the belief that clear-cut physiologic observations were lacking to clarify the basic issue as to whether or not new nerve fibers could grow into carcinomatous tissue and keep pace with it for a specific function, the effect of nerve stimulation upon tumor blood vessels was studied.

MATERIALS AND METHODS

Anterior chamber transplants of the Brown-Pearce carcinoma in the rabbit eye and a mouse mesothelioma (MT_8) in the guinea pig eye were utilized. Transplantation of tissue into the anterior ocular chamber was made by the method of Greene (5). Transplants were made into only one eye of each animal, and their blood vessels studied by a slit-lamp and a corneal microscope at a magnification of 30 and 70.

Under nembutal anesthesia, the cervical sympathetic cord ipsilateral to the transplanted eye was isolated and transected at a level approximately 2 cm. below the cervical ganglion. Stimulation of the proximal transected cord with alternating current was effected by means of a Harvard inductorium and a single dry cell. The period of stimulation lasted seconds. Blanching and retraction of the eyelids were used to check upon the presence of adequate stimulation.

These experiments employed 57 rabbits, averaging 2.5 kg. in weight, and 47 young guinea pigs, averaging 500 gm. in weight. The per cent take of the Brown-Pearce carcinoma in the rabbit eye was 100 per cent, and the tumors grew rapidly, filling the anterior chamber, and perforating through the cornea between the seventh and tenth day of growth. Inasmuch as the earliest recorded observation of a nerve-supplied blood vessel contracting upon stimulation has been after 8 days of growth (2), it was deemed inadvisable to examine any of the tumors before the eighth day, since negative results could not necessarily mean that functioning nerve fibers would not grow into tumor tissue. This decision meant the loss of 22 rabbit tumors due to perforation. Spontaneous death, anesthetic death, corneal opacity, sacrifice for transplantation, and use for photography accounted for an additional 22 rabbits. Therefore, only 13 rabbit tumors were available for study.

The MT_8 grew slowly in the guinea pig eye, and observations were made from the fourteenth day to the thirty-fourth day of growth, usually shortly after the twentieth day of growth. A 61 per cent take, corneal opacity, spontaneous death, and sacrifice for transplantation left 22 MT_8 tumors available for observation.

Histologic studies were made on sections stained with hematoxylin and eosin, Masson-Goldner's stain (4), Van Gieson's stain, and by Bodian's protargol method for nerve fibers. All eyes were fixed either in Bouin's fluid or glacial acetic-formol-alcohol fluid, and imbedded either in celloidin or paraffin.

Macroscopic stereophotographs were taken with a 5×7 inch view camera equipped with a one-inch lens. Exposure was made on fast panchromatic film by "open flash" technique with the lens set at f. 16. The light was polarized by the insertion of a sheet of neutral polaroid material. Surface reflections were largely removed by a polaroid filter over the lens.

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RESULTS

The tumor blood vessels seen through the cornea are readily visualized (Fig. 3); thus, a secondary purpose of this paper is to call attention to the value of anterior chamber transplants as a tool to study the vascularity of malignant growths. The Brown-Pearce carcinoma was the better vascularized of the two tumors and was easier for

FIG. 1.—Section showing arteriole just beneath cornea in a 22-day MT_8 tumor. Mag. × 250.

study inasmuch as the cornea did not undergo vascularization. The MT_8 tumor invariably elicited an ingrowth of vessels into the cornea, and derived part of its blood supply from vessels arising at the sclerocorneal junction. Careful focusing of the slit-lamp microscope to distinguish the vessels which lay on the surface of the tumor from those which lay deep in the cornea was necessary. The extensive vascularization seen on the surface of the Brown-Pearce tumor appeared for the most part to be composed of richly anastomosing capillaries; whereas the comparatively sparse circulation on the surface of the much longer growing MT_8 tumor appeared largely to be on the venous side. Decision as to whether a given vessel was a capillary, vein or artery was based on caliber, color, tortuosity, and flow of blood into or out of the tumor; however, regardless of type, all vessels were closely watched for a response to nerve stimulation. Except for one questionable instance, rhythmical active vasomotion was not seen. It is possible that the anesthesia obliterated this activity (10).

Upon sympathetic stimulation, the following observations on the 13 Brown-Pearce transplants were obtained. Blanching (the tumor paled and the vessels assumed a slight bluish hue) definitely occurred in 9 of the tumors, and was not seen in 4. Contraction of vessels was difficult to observe, as will be further explained, but was seen to occur in 1 or 2 vessels of 4 of the transplants (Fig. 4), was considered questionable in 2, and was not seen in 7.

In the 22 MT_8 tumors blanching was found in 18, was considered questionable in 3, and was not seen in 1 tumor. Contraction of a vessel was seen

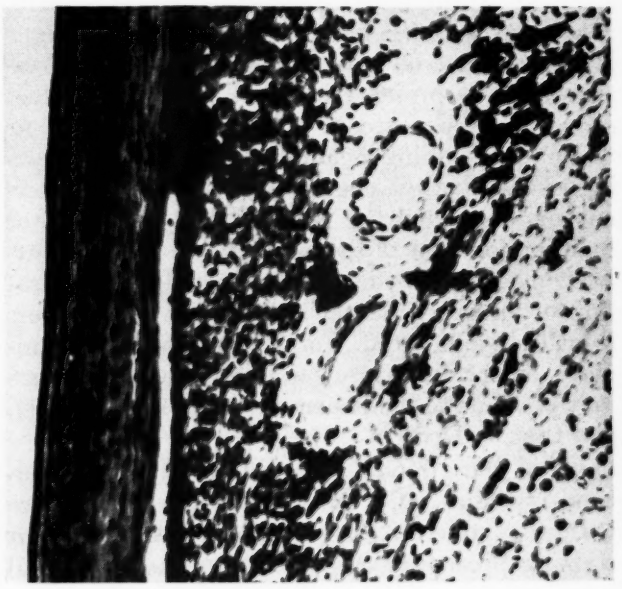

FIG. 2.—Section showing arterioles just beneath cornea in a 26-day MT_8 tumor. Mag. Slight reduction from × 250.

in 4 MT_8 tumors, was considered questionable in 2, and was not found in 15.

The time of contraction varied from a second to 5 or 6 seconds, and the reduction in diameter varied from 50 to 100 per cent. The contracted state persisted for lengths of time from a few seconds to 20 seconds or more, depending somewhat upon the strength and duration of the original stimulus. Dilatation followed the contracted state. These definite contractions could be repeated as frequently as stimulation was applied.

Histologic studies revealed a richer network of

capillary loops in the Brown-Pearce tumor than in the MT_8 tumor, and a small number of well-differentiated arterioles in both. These arterioles were usually found in the deeper parts of the tumor, closest to the iris from which the tumors derived their blood supply; however, even in the random sections studied, an occasional arteriole could be found just below the cornea on the surface of the tumor (Fig 1 and Fig. 2).

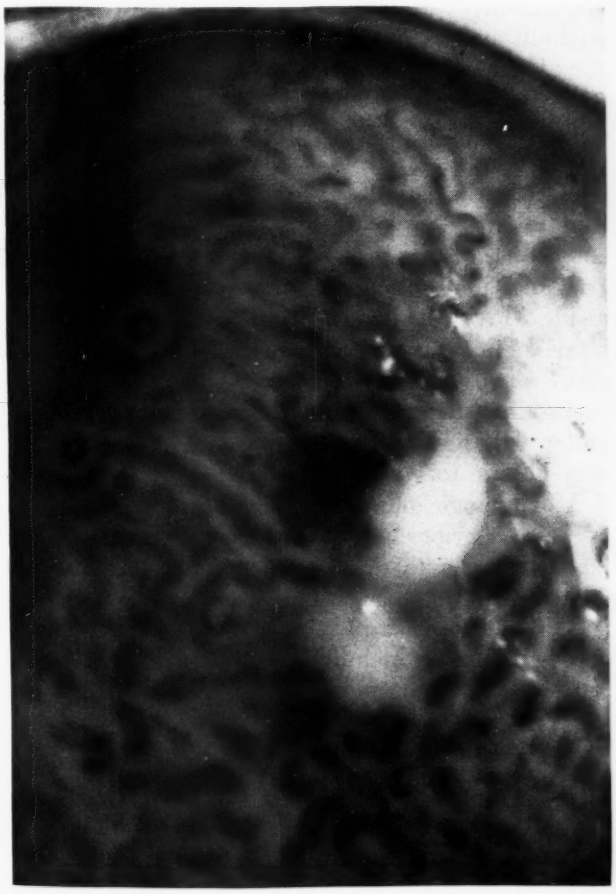

FIG. 3.—Photograph taken through the cornea showing extensive vascularization of a 9-day Brown-Pearce tumor in vivo. Dark linear areas represent vessel loops. Mag. Slight reduction from × 60.

Bodian's protargol method revealed nerve fibers coursing through both tumors either singly or in loosely knit bundles of a dozen or more. In view of the youth of the transplants (a number were only 9 days old) the number of fibers was surprising. Although these were random sections, it was the opinion of several consultants that nerve fibers had been demonstrated in the tumors (Fig. 5).

DISCUSSION

The only vessels visualized by the technique employed are those on and just below the surface of

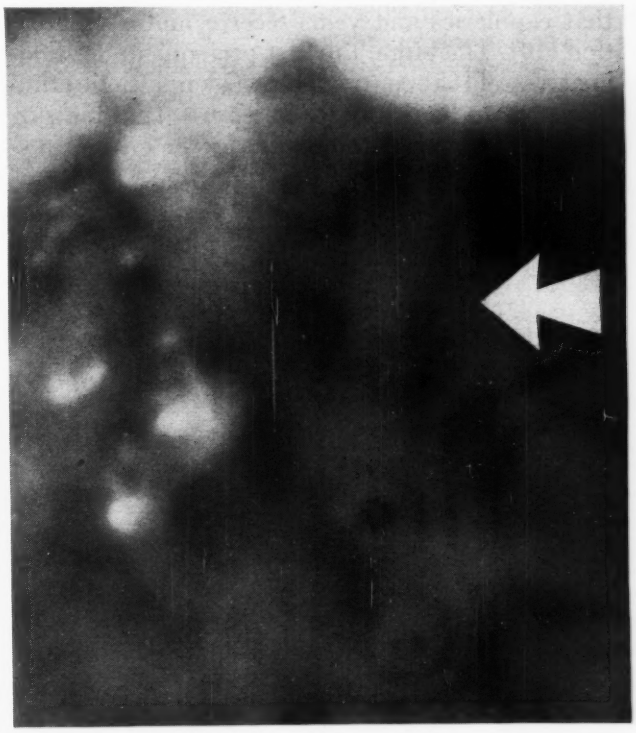

FIG. 4.—a) Photograph taken at end of stimulation. Mag. × 150.

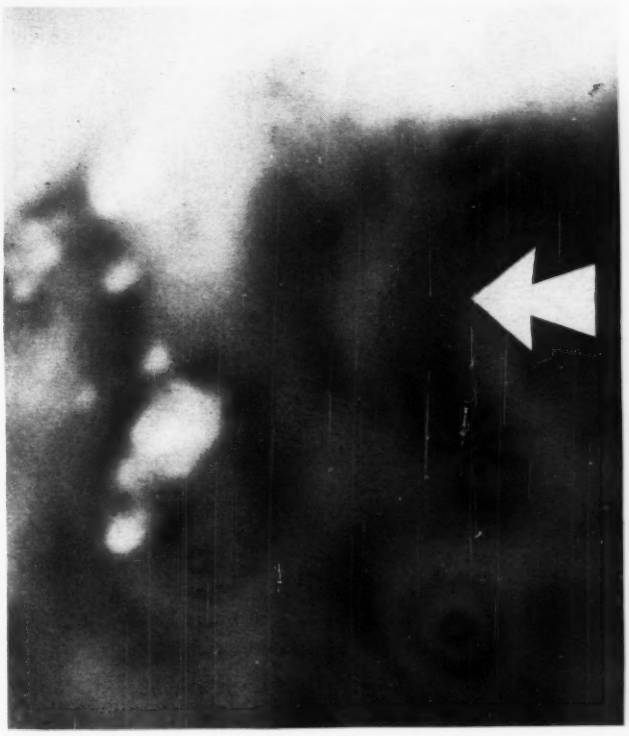

FIG. 4.—b) Photograph of same field after a minute recovery period. Mag. × 150.

the tumor. At present, there is considerable doubt that capillaries and veins receive motor innervation (10). Therefore, the first prerequisite for finding a vessel that contracts upon sympathetic stimulation is to have an arteriole present on the surface of the tumor. Although the few arterioles present in the tumor are mostly located in the deeper regions of the tumor near the origin of the blood supply, they can rarely be found on the surface. These anatomical facts explain in part the preponderance of negative findings.

FIG. 5.—Section shows loosely knit bundle of nerve fibers in 8-day Brown-Pearce tumor. Bodian's protargol stain. Mag. Slight reduction from × 750.

Clark, Clark and Williams (2) report that the extent of definite active contractility in a growing arteriole is governed by the extent of growth of the nerve supplying the arteriole. Hence, it is possible for an arteriole to be present on the surface of the tumor, and not show a response to stimulation until the supplying nerve reaches the segment under observation. These same authors report seeing, in double-walled transparent chambers inserted in the rabbit's ear, newly formed arterioles that took as long as 3.5 and 7.5 months to develop definite contractility, and many newly formed arterioles without nerve supply. Thus, in non-malignant tissue, there is a variable degree of innervation of newly formed arterioles. These are presumably the additional factors accountable for the paucity of positive findings.

Blanching cannot be considered indicative of functioning nerves within the tumors, since it could possibly be brought about by contraction of arterioles just outside the tumor, as well as those inside. However, it does demonstrate a vasomotor influence on malignant growths. This is in agreement with Willis's suggestion that the reported variations in rate of growth of tumors in tissues under different states of disturbed innervation may be due to vasomotor changes.

Morphologic demonstration of nerve fibers within these anterior chamber transplants is considered especially significant since these tumors, so to speak, grew in space. Therefore, it appears unreasonable to assume that the fibers represent nerves becoming included within the cancer owing to the invasive character of its growth.

SUMMARY

Tumor blood vessel contraction in Brown-Pearce carcinoma and mouse mesothelioma (MT_8) anterior chamber transplants results from sympathetic nerve stimulation and nerve fibers within these tumors have been demonstrated.

These observations imply that functioning nerve fibers can grow into cancerous tissue. These experiments further suggest that the reported variations in rate of growth of tumors in tissues under different states of disturbed innervation may be due to vasomotor changes.

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The Tissue Transplant Technic as a Means of Testing Materials for Carcinogenic Action*†

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Recent experimentation has shown that carcinomas and benign and malignant papillomas develop from epithelial tissues transplanted from mouse embryos into adult animals along with methylcholanthrene dissolved in olive oil (1–4). The original experiments had been undertaken to find out whether the epithelial tissues of embryos possess the potentiality to become cancerous, or whether this potentiality is acquired later in life. When the fact emerged that epithelial tumors could be counted upon to appear quickly and regularly from the tissue transplants, the question arose as to whether this method could be employed as a means of testing chemical compounds for carcinogenic action upon selected tissues. The experiments now reported were designed to provide information on this point by tests with several carcinogens in addition to methylcholanthrene.

A genetically homogeneous strain of mice must be used in order to assure that the transplanted tissues will grow in all of the new hosts. In our hands, the C strain has proved by far the most satisfactory. The tissues of embryos of other strains of considerable genetic homogeneity (A, C3H, I) tolerate the exposure to methylcholanthrene less well and many implants die (2–4).

* This work was supported in part by a grant from the Office of Naval Research.

† Presented in part at the fortieth annual meeting of the American Association for Cancer Research, Detroit, 1949.

METHODS

A full description of the technic has been presented in previous papers (1, 3, 4). The tissues are taken from mouse embryos near term (18 to 22 mm. in length), removed under aseptic precautions, and killed by cutting off their heads. The desired embryo tissue or organ is minced in Locke's solution, and for each implantation 0.05 cc. of this tissue suspension is drawn into a syringe, then 0.05 cc. of the carcinogen solution is drawn up into the same syringe, and injection done into the posterior thigh muscles of adult mice through skin incisions made over the calf muscle. The edge of the skin incision is wiped with 1/1000 solution of bichloride of mercury before introduction of the needle to avoid the carrying in of epithelial cells of the host's skin. Quarter cc. Luer syringes fitted with 16 gauge needles are used. Implantation is facilitated by filing down the bevel of the needle to shorten it. The skin incision must be pinched together with the fingers over the needle during and for a few seconds after injection to prevent backflow and escape of oil.

Olive oil (U.S.P.) was generally employed as the solvent, and solutions were prepared in it of market-

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All sections were stained with phloxin and methylene blue. Mr. R. F. Carter made the photographs. Figs. 1 to 5.—Tumors induced by implantation of mouse embryo epidermis into the leg muscles of adult mice together with 3:4-benzpyrene.

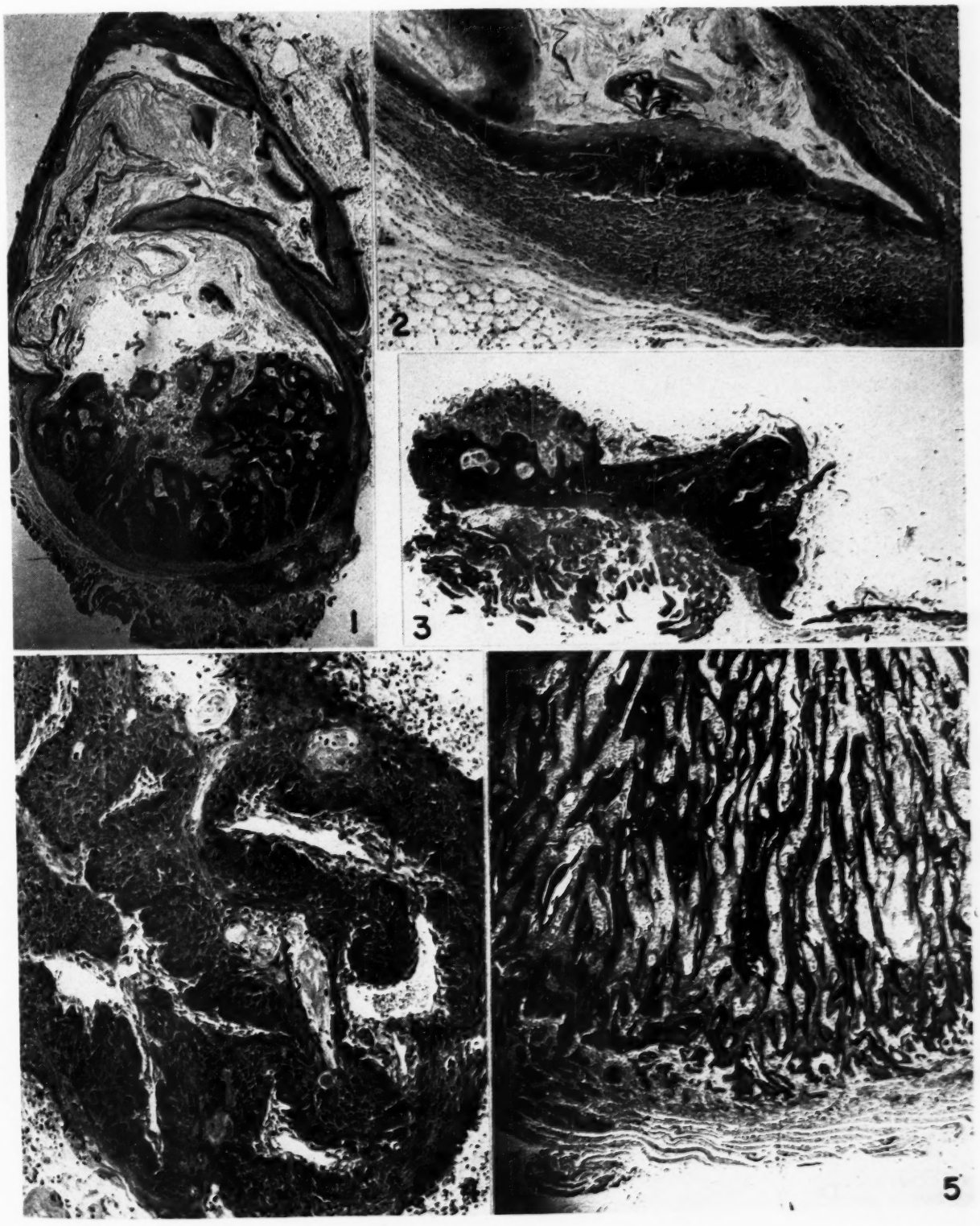
FIG. 1.—Cross-section through a cyst removed 141 days after implantation. The lower third of the cyst cavity is filled with a malignant papilloma. Elsewhere the cyst wall is thin, but slightly hyperplastic and devoid of hair follicles and sebaceous glands, such as form in cysts not exposed to a carcinogen. Note the orderly lamellae of keratin over the thin regions of the cyst wall. Over the tumor the keratin was jumbled and pultaceous. 17.5 ×.

FIG. 2.—Early stage showing origin of a carcinoma from a cyst wall. The section shows the area between the arrows in Fig. 1. The epithelium has heaped up and begun to grow down. Toward either side of the growth the wall is merely hyperplastic. 79 ×.

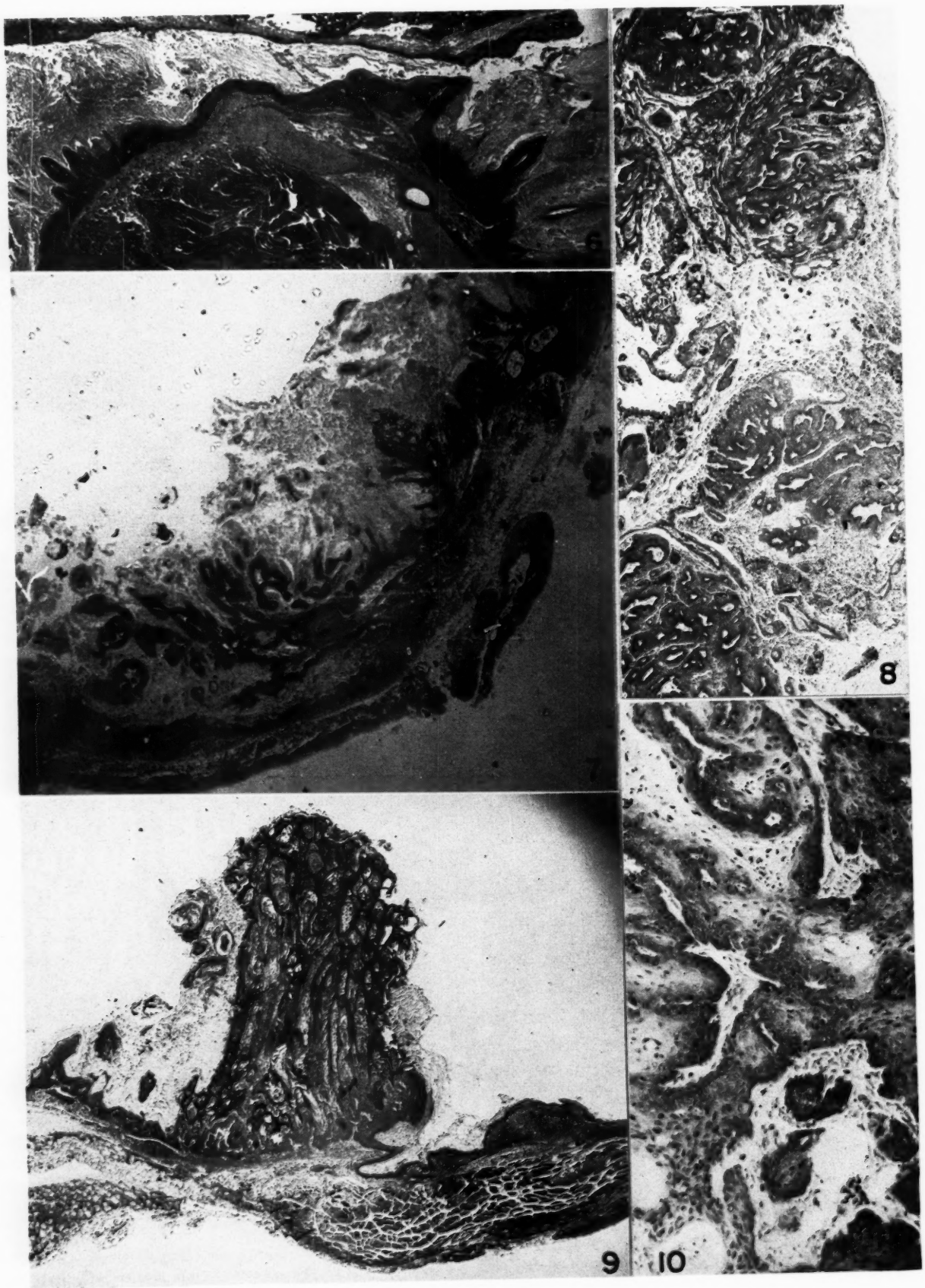
FIG. 3.—Section of wall of a cyst found 114 days after implantation. The cyst was 12 mm. across, lined for the most part by a shallow layer of epithelium such as the strip shown at the right of the photograph. In one area the wall was thickened and rugose. The photograph shows this thickened area, which contains pearls and forms an irregular border with the subjacent connective tissue and muscle. Pieces from this area gave rise to large tumors upon transplantation to new hosts. 17.5 ×.

FIG. 4.—Detail of tumor found 29 days after transplantation of a piece of the growth shown in Fig. 3. The tumor was a cyst 17 mm. in diameter with rugose walls up to 4 mm. thick. The histology and rapid growth of this tumor establish it as a squamous cell carcinoma. There are many hyperchromatic nuclei and occasional mitoses. × 110.

FIG. 5.—Thick-walled cancer cyst found 104 days after implantation of embryo epidermis with benzpyrene. Downgrowths from the base have everywhere invaded the underlying tissue. × 17.5.



FIGS. 1–5



FIGS. 6–10

grade samples of the following carcinogens in the concentrations stated:

20-methylcholanthrene (Edcan)	1%
3:4-benzpyrene (Hoffman, LaRoche)	2%
3:4-benzpyrene (Hoffman, LaRoche)	1%
1:2:5:6-dibenzanthracene (Edcan)[1]	1%
1:2:5:6-dibenzanthracene (Edcan)	⅔%

The necessity for special purification of most of these chemicals was only secondarily recognized and then carried out. For certain of the tests, tricaprylin was used as the solvent instead of olive oil. The tricaprylin employed was the generous gift of Dr. Carl Larsen. It was a colorless liquid with melting point 8.2 to 9.2° C.

All solutions were distributed in 1 cc. quantities into test tubes, which were autoclaved and stored in the icebox. They must be warmed to redissolve the carcinogens before use. The carcinogens are referred to hereafter by the abbreviations: MC, BP, and DBA.

FINDINGS

Experiments 1, 2, and 3 were carried out with the olive oil solutions of market-grade carcinogens. For each experiment skin from a separate litter of embryos was utilized. For each injection, 0.025 cc. of oil containing a carcinogen was drawn into the syringe, then 0.075 cc. of Locke's solution, then 0.025 cc. of tissue suspension. The animals were examined at weekly intervals after implantation and chartings made of the size of the nodules at the implant sites, as judged by palpation. As shown in previous papers, a doubling of the size of a nodule within a week can usually be taken as the date of onset of malignant change. The animals were allowed to live several weeks after such sudden enlargement of the nodules in order to get tumors sufficiently big and invasive to offer indubitable evidence at autopsy of their neoplastic character. Nodules that increased suddenly in size within 80 days after implantation invariably proved to be carcinomas at eventual autopsy. Some that showed sudden increase later than 80 days were also found to be carcinomas, but others in this group of relatively late change showed both cancer and sarcoma and still others only sarcoma.

The cancers were cystic growths, often 15 to 30 mm. in diameter, filled with mushy, pultaceous keratin and fluid, and lined by thick, rugose walls adherent to the surrounding tissue, sometimes surrounding the femur and destroying the bone or sweeping up to invest the pelvis in masses of firm tissue that pushed into the abdomen. Many had papillomatous characteristics (See figures). Blocks were taken of all growths at autopsy, and the diagnosis of cancer as listed in the tables has been in each instance supported by histological study. Photographs of carcinomas induced in this manner by methylcholanthrene have already been published, and many successful transplantations attest their neoplastic character (1, 3, 4). In the present work, successful transplantations were carried out with cancers induced by benzpyrene and dibenzanthracene (Figs. 4, 7, 10).

At implant sites that failed to yield tumors, clusters of tiny flattened creamy cysts, each only 2 or 3 mm. in size were found. These little cysts had paper-thin walls and were filled with dry, lamellated keratin. They gave no suggestion of malignant change, except in 6 instances where one cyst in a cluster was larger than the others, firm, spherical instead of flattened, and had a thickened wall. Sections showed that the thickening was epithelial in nature and downgrowths indicated beginning malignancy. These border line cases are not listed as cancer in the tables. The animals were not allowed to live out their natural lives, but were killed at a maximum of 6 months after implantation. Cancers might conceivably have arisen at additional sites had the mice been kept longer.

The sarcomas were solid growths without the cystic character of the cancers. In 10 instances a

[1] This amount of dibenzanthracene was not completely soluble in olive oil. By cooling immediately after autoclaving, the crystals that did not go into solution remained fine enough to pass easily through the needle at the time of injection.

FIGS. 6 TO 9.—Tumors induced by implantation of mouse embryo epidermis into the leg muscles of adult mice together with 1:2:5:6-dibenzanthracene.

FIG. 6.—12 mm. cyst found 73 days after implantation. The cyst has been collapsed. Half its surface, as shown at the top, was lined by only a shallow layer of epithelium. Over the other half, the epithelium was greatly thickened and thrown up into papillomatous projections. × 17.5.

FIG. 7.—Detail of growth found 76 days after transplantation of a piece of the papillomatous tissue shown in Fig. 6. The new tumor was a cyst 16 mm. across lined everywhere with disorderly and invasive papillomatous tissue, as shown. × 17.5.

FIG. 8.—Squamous cell carcinoma found 108 days after implantation of embryonic epidermis with dibenzanthracene. The tumor was 30 mm. in diameter and had ulcerated through the overlying skin. Great fleshy masses of tumor tissue replaced the leg muscles and swept up to invest the pelvis. Transplants of this tumor grew rapidly and killed new hosts (cf. Fig. 10). × 17.5.

FIG. 9.—Papilloma based upon a cyst wall 203 days after implantation of embryonic epidermis with dibenzanthracene. Its dry, horny character indicates that it long existed as a benign growth, but its fleshy base has begun to show downgrowths and is replacing the cyst wall toward the left. The thin cyst wall to the right illustrates the discrete origin of the tumor. × 17.5.

FIG. 10.—Section of tumor got by transplanting a piece of the growth shown in Fig. 8. The tumor in the new host was 25 mm. in diameter when the mouse was killed 27 days after transplantation. × 108.

cancer cyst was found embedded in and overgrown by a sarcoma. Since there was no way of determining whether the sarcomas arose from implanted embryonic cells or from the connective tissues of the host, their occurrence is of no interest as bearing on the use of the tissue transplant technic for assaying the carcinogenic action of a compound against a transplanted tissue.

In the first 3 experiments, a total of 104 mice were employed, 91 of which survived for more than 50 days after implantation. The data from these 91 animals are summarized in Table 1. They had received a total of 138 implants, some having been injected in only 1 instead of both hind legs. It should be emphasized that the carcinogens used in these 3 experiments were ordinary market-grade chemicals. Methylcholanthrene was the only one kill the tissue implanted with them, for autopsies disclosed well-formed epithelial cysts. But the cysts had failed to enclose the oils, which lay outside in the muscles. This led to a comparative study, to be reported elsewhere, in which it was found that purified dibenzanthracene, and to a lesser extent benzpyrene, lacked the ability of methylcholanthrene to attract epithelial cells to grow toward and surround globules of oil containing them.

The suprisingly small yield of tumors with "dibenzanthracene" in the foregoing tests may have been due in part to the poor quality of the chemical employed, for our batch instead of being white had a bright orange color. According to Fieser, orange-colored preparations of dibenzanthracene may contain up to 80 per cent of a non-

TABLE 1

EFFECTS OF MARKET-GRADE METHYLCHOLANTHRENE (MC), BENZPYRENE (BP) AND DIBENZANTHRACENE (DBA) ON IMPLANTED SKIN
EXPERIMENTS 1, 2, AND 3

		Findings at Implant Sites					Time between Implantation and		
Carcinogen	No. of implants	Carcinoma	Carcinoma and sarcoma	Sarcoma	Benign cysts	No growth	Sudden growth of nodule* (days)	Autopsy* (days)	Percent of implants yielding carcinoma**
1% MC	33	22	4	4	3	0	44–96	83–107	78.9
1% BP	31	17	3	2	9	0	80–122	104–181	64.5
2% BP	22	5†	4	6	6	1	98–119	114–163	40.9
2/3% DBA	25	4	0	0	21	0	71–161	85–182	16.0
1% DBA	27	2	0	2	21§	0	79–183	104–183	7.4

* These figures refer only to sites at which carcinoma developed (Column 3).
** Includes sites at which cancer occurred alone or with sarcoma (Columns 3 and 4).
† Two of these tumors were transplanted and grew rapidly, killing the new hosts.
§ Two cysts contained benign stalked papillomas.

that approached purity, a fact that goes far to explain the relatively poor results got with the other two.

From Table 1, it can be seen that in the 33 implants where the tissue was exposed to methylcholanthrene, carcinomas arose in 22 instances, carcinoma and sarcoma in 4. Carcinoma was thus induced at 26 of the 33 sites, or 78.9 per cent. Where the tissue was put with 1 per cent "benzpyrene," carcinomas resulted at 64.5 per cent of the implant sites, and appeared later than did those induced by methylcholanthrene. The quotation marks are inserted because of the subsequently recognized impurity of the chemical. The yield of cancers with 2/3rd per cent "dibenzanthracene" was even less, 16 per cent.

An unexpected finding was that increase in the concentration of "benzpyrene" or "dibenzanthracene" reduced the incidence of carcinomas got with these materials to 40.9 per cent and 7.4 per cent respectively. The stronger solutions did not carcinogenic impurity (5). Similarly, our "benzpyrene" had a greenish cast and a melting point 10 degrees below that given in the literature. Through the cooperation of a manufacturer, we had a batch of dibenzanthracene submitted to the lead tetraacetate purification procedure (5) and obtained a white crystalline substance. From the same source we obtained purified benzpyrene and methylcholanthrene.[2] As a gift from Dr. J. Engelbreth-Holm we received a supply of 9:10-dimethyl-1:2-benzanthracene. All four chemicals were crystalline and their melting points corresponded sufficiently well with those in the literature to assure a high degree of purity (Table 2). Though olive oil had proven satisfactory as a solvent, it contains heterogeneous and not completely known constitutents, and different lots doubtless vary in composition. For this reason the pure substance tricaprylin was selected as a solvent (See "Methods").

[2] The chemicals were prepared by the Edcan Chemical Corporation, Norwalk, Connecticut.

A further consideration deserves attention in comparing carcinogens for their ability to call forth tumors. It has been the general practice to employ solutions of equal percentage composition by weight. A more exact procedure would be to compare the carcinogens molecule for molecule rather than milligram for milligram, and for this purpose equimolar solutions of the several carcinogens were prepared for the next experiments. The maximum solubility in tricaprylin of the least soluble compound, dibenzanthracene, was ascertained and all compounds were made up in this concentration, which amounted to 0.02 molar. The differences between solutions prepared on a percentage by weight basis and those prepared on an equimolar basis are shown in Table 2.

Experiment 4 was carried out with skin from a litter of 6 embryos, 18 mm. long, implanted into one leg of 70 adult C mice together with tricaprylin as

TABLE 2

Carcinogen	Melting points: Our materials	Melting points: From literature*	Molecular weight	Amount present per cc. in 0.5% solution mgs.	Amount present per cc. in 0.02M solution mgs.	Solution by weight equivalent to 0.02M solution
1:2:5:6-dibenzanthracene	264–266° C.	266–266.5° C.	278	5.0	5.56	0.556%
20-methylcholanthrene	178–180	179.5–180	268	5.0	5.36	0.536%
9:10-dimethyl-1:2-benzanthracene	122–124	122–123	256	5.0	5.12	0.512%
3:4-benzpyrene	177–178	178.5–179	252	5.0	5.04	0.504%

* Fieser, L. Am. J. Cancer, **34**:37–121, 1938.
Bachmann, W., and Chemerda, J. J. Am. Chem. Soc., **60**:1023–1026, 1938.

such or tricaprylin containing 0.02 molar methylcholanthrene, benzpyrene, dibenzanthracene, or 9:10-dimethyl-1:2-benzanthracene. The purified chemicals just described were employed for this and subsequent work. Since we had previously dealt with 1 per cent solutions and since the 0.02 M solutions now used were approximately 0.5 per cent, the volume injected was doubled. Each inoculum consisted of 0.05 cc. of one or the other tricaprylin preparation and 0.05 cc. of the tissue suspension.

There were 14 mice in each group, 8 of which were killed within 21 days after implantation for another purpose. Six animals were thus left in each group to be followed for development of tumors. The findings in these are given in Table 3.

Tumors appeared with unusual rapidity in the methylcholanthrene group, all 6 animals having microscopically proven cancers within 73 days after implantation, when the last members of the group were killed. The earliest cancer was found on the forty-fifth day. The animal bearing it had a nodule 8 mm. across by the twenty-eighth day. By the forty-fifth day, when the animal was killed, this nodule had become a firm mass 15 mm. in diameter. It was a cyst with rugose walls up to 2 mm. thick. Pieces of it transplanted into 2 new hosts gave rise to huge, thick-walled cancer cysts.

In the dibenzanthracene group, tumors arose far more slowly. Only on the seventy-second day did any animal give evidence of tumor, as judged by palpation. At this time, one of the 6 mice had a firm mass 12 mm. in diameter. It was killed and a cyst found which was replaced over half its surface by a papillomatous growth up to 3 mm. thick (Fig. 6). Pieces of it transplanted into 2 new hosts gave rise to a large malignant cyst in one of them (Fig. 7). After 79 days, another animal of the original group exhibited a firm mass that had suddenly increased in size. Similar masses appeared in 2 more mice by the 98th day. In all 3 the growths enlarged progressively and thick-walled cancer cysts were found when the animals were killed two or three weeks later. Transplants from two of them reproduced in new hosts growths resembling the original carcinomas (Figs. 8 and 10).

Of the remaining 2 mice in the dibenzanthracene group, one was killed with a large sarcoma in the leg on the hundred and thirty-sixth day. The nodule at the implant site in the other remained remarkably constant in size until the hundred and forty-fifth day when it doubled in size in the space of a week and thereafter enlarged steadily. The animal was killed on the hundred and seventy-fifth day. The mass was a cyst, of which two-thirds of the surface had been replaced by a rugose growth that proved microscopically to be carcinoma.

Of 7 mice given benzpyrene, 6 developed cancers at the implant sites, the seventh yielding only small benign cysts even though allowed to live for 254 days. As shown in Table 3, the cancers came at approximately the same time as in the dibenzanthracene group.

The 9:10-dimethyl-1:2-benzanthracene proved too toxic in the concentration used. It killed the epithelial tissue implanted with it and caused much inflammation and formation of scar tissue. A sarcoma arose at 1 of the 4 sites where it had been put.

No tumors arose in the control group given merely skin and the solvent, tricaprylin.

This test with the purified carcinogens dissolved in tricaprylin gave far better results than the preceding work; nevertheless, many materials submitted for test for carcinogenicity would manifestly have less potency than the powerful carcinogens

thus far studied. Hence it was desired to find out whether the method was in fact useful in the case of weak solutions of a carcinogen, and, if possible, to increase the responsiveness of the tissue.

A concentration of dibenzanthracene reduced to an amount giving only an occasional tumor was selected for the next experiment. As already mentioned, globules of oil containing dibenzanthracene fail to attract epithelial cells, hence this carcinogen has less chance of becoming incorporated into the cysts formed by the implanted epidermal cells. For this reason we returned to a device used in earlier work, namely Scharlach R (Grübler) was added to the dibenzanthracene preparation in the view that the dye would attract epithelial cells to grow around globules of oil containing it and thereby effect encystment of the dibenzanthracene. It was also felt that the stimulating effect of Scharlach R might afford some further advantage, but a more promising co-carcinogen, croton oil, came to mind through the work of Berenblum (6). The following test was therefore made.

Experiment 5 was performed with the skin of 7 embryos 18 to 19 mm. long. It was put in both hind legs of 23 male C mice together with 0.05 cc. of one or another of the following solutions made up in olive oil (our supply of tricaprylin being exhausted):

DBA: 0.01 M dibenzanthracene in olive oil

DBA+SR: 0.01 M dibenzanthracene in olive oil containing 2% Scharlach R

DBA+SR+CO: 0.01 M dibenzanthracene in olive oil containing 2% Scharlach R and 0.1% croton oil.

SR+CO: Olive oil containing 2% Scharlach R and 0.1% croton oil

Where the tissue was put simply with 0.01 M dibenzanthracene, cancers occurred at only 3 out of 12 sites and became manifest only after a comparatively long time, 140 to 202 days (Fig. 9). This small and late yield of tumors contrasted with the results of Experiment 4, where twice as strong a solution of dibenzanthracene had led to cancers at practically every implant site. The 0.01 M solution (approximately 0.25 per cent by weight) employed in the present test could therefore be considered as providing a minimal effective dose (0.14 mgm. at each site). At sites where cancer failed to appear, there was evidence that the

TABLE 3

EFFECTS OF 0.02 MOLAR SOLUTIONS OF PURIFIED CARCINOGENS ON IMPLANTED SKIN
EXPERIMENT 4

Mouse no.	Carcinogen	Time between Implantation and Sudden growth of nodule (days)	Time between Implantation and Autopsy (days)	Findings
1	MC	45	45	Malignant papilloma (grew rapidly and progressively on transfer)
2	"	43	58	Squam. carc.*
3	"	50	73	Squam. carc.
4	"	58	73	Squam. carc.
5	"	73	73	Malignant papilloma
6	"	73	73	Squam. carc.
	Average:	57	65.8	
7	DBA	72	73	Malignant papilloma (grew slowly but progressively on transfer)
8	"	79	108	Squam. carc. (grew rapidly and progressively on transfer)
9	"	98	108	Squam. carc.
10	"	98	115	Malignant papilloma (grew but regressed on transfer)
11	"	(119)	(136)	*Sarcoma*
12	"	145	175	Squam. carc.
	Average:	101.8	119.2	
13	BP	79	108	Squam. carc. and a malignant papilloma
14	"	98	108	Squam. carc. and two benign papillomas
15	"	108	115	Squam. carc.
16	"	108	115	Squam. carc.
17	"	108	136	Squam. carc.
18	"	158	175	Squam. carc.
19	"		(254)	*Benign cysts*
	Average:	109.8	126.1	

* Squam. carc. = squamous cell carcinoma.

oil had failed to become enclosed into the cysts. At one such site a big reservoir of oil was found nestled directly against the wall of a large cyst, yet sections showed that the epithelium had made no effort to grow out and enclose it. At the other sites, multiple small cysts were found and no oil could be detected when their dry, keratinized contents were broken up in water.

DBA+SR.—Among 12 implants with this material, only 2 tumors occurred: a benign papilloma in an animal dead after 109 days, a cancer in an animal killed on the hundred and twenty-first day. The animals bearing the other implants died between 110 and 140 days of a bronchopneumonia endemic in this colony. Their growths were merely benign cysts with nothing to suggest tumor either on gross or histological examination. This smaller yield of tumors may have been due to the poor condition and relatively early death of the animals. It was certainly not due to any failure of exposure to the carcinogen, for autopsies showed that the red-colored oil had been almost regularly and almost completely enclosed within the cysts. Only at 2 of the 12 sites did it lie outside. In these 2 instances the oil depots were found at a distance from the cysts, shut away from them by intervening muscle bundles, doubtless a result of accidental jerking back of the needle at the time of injection so that the oil was put in one place and the tissue deposited at another.

DBA+SR+CO.—With this material tumors were got at 5 out of 9 sites, and these appeared within 87 to 121 days. There were cancers at 4 sites and a benign papilloma at the fifth. A tenth site had been implanted and a cyst had formed there, but it had died after engulfing all the red oil. The oil was wholly enclosed in the cysts except at one site where it lay at a distance. Some tumors in this group are shown in Figures 12 and 13. There were more of them than in either of the preceding groups, and they appeared more quickly, and this despite the fact that the animals bearing them had lost much weight as result of bronchopneumonia and were almost moribund when killed.

The control group implanted with skin together with our preparation of Scharlach R and croton oil (SR+CO) gave only benign thin-walled cysts. An occasional cyst exhibited a hyperplastic wall (Fig. 11), but the majority were lined merely with flattened epithelial cells in a shallow layer only 2 to 3 cells deep. The croton oil used in these experiments was supplied by an American firm (Magnus, Mabee, and Reynard) and is said to have less tendency to produce hyperplasia and promote tumors than croton oil manufactured in England (7).

Because of the endemic pneumonia in our stock of C mice, we obtained a subline of this strain from Dr. H. B. Andervont. Skin, gastric and pulmonary tissue transplanted from embryos of Andervont's mice into adults of his stock were found to grow and survive for months at every implant site. Skin and pieces of the squamous por-

FIG. 11.—Section of cyst wall 140 days after implantation of embryo epidermis with olive oil containing 2 per cent Scharlach R and 0.1 per cent croton oil. The epithelium is moderately hyperplastic and underlain by a slight accumulation of round cells. There are no hair follicles or sebaceous glands. × 116.

FIG. 12.—Cyst wall 100 days after implantation of embryo epidermis with dibenzanthracene dissolved in olive oil containing Scharlach R and croton oil. The wall is composed of only a few layers of squamous cells except in the middle of the photograph, where there is a small mound of heaped-up epithelium from which cords of cells have grown down into the subjacent tissue, a beginning carcinoma. The recent onset of the neoplastic change is mirrored in the pattern of the keratin, which appears granular immediately over the little tumor and elsewhere is made up of orderly lamellae. Slightly reduced from × 34.

FIG. 13.—Papilloma seated upon a cyst wall 100 days after implantation of the same materials as in Fig. 12. Slightly reduced from × 17.5.

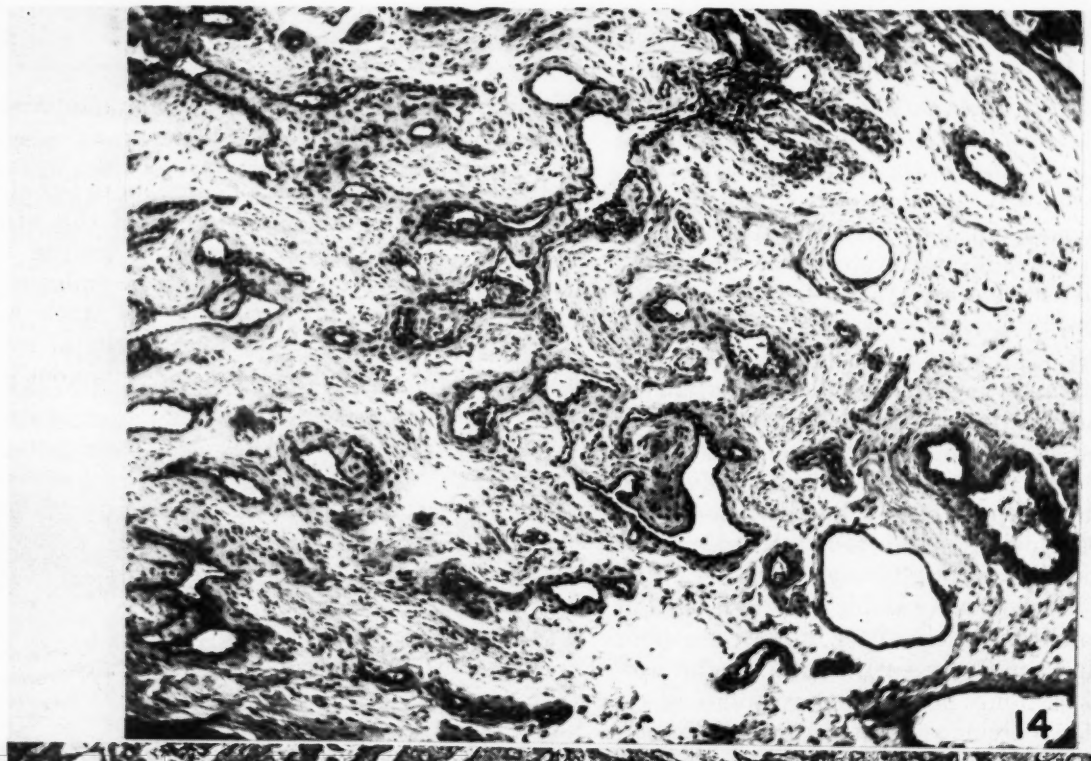


FIG. 14.—Adenoacanthoma found 178 days after implantation of pieces of the glandular part of mouse embryo stomachs together with dibenzanthracene. The tumor was an almost solid growth, 28 mm. in diameter. There was no formation of keratin, but the epithelium heaped up into stratified layers of flattened cells which desquamated singly. This tumor has preserved its histological character on transfer through two generations of new hosts. × 108.

FIG. 15.—Section through growth found 139 days after implantation of mouse embryo lungs with dibenzanthracene. The majority of the growth was the squamous cell carcinoma shown at the top of the photograph, pieces of which gave rise to large rapidly growing tumors in new hosts. At the left of the photograph is a big area of loose alveolar tissue with the histology of lung tissue. In the midst of it there is a compact adenoma (Block A) resembling those seen in adult lungs. In 10 discrete spots, the alveolar walls have undergone squamous metaplasia and small tufts of squamous tissue are seated upon them (Block B). × 20.

tion of the stomach were implanted with olive oil containing 1 per cent methylcholanthrene and 2 per cent Scharlach R. Both types of tissue survived well in contact with these chemicals, and went on to form cancers, several of which were transplanted and grew in every new host. The genetic homogeneity of this subline and its usefulness for our purpose was thus established, and we have replaced our old strain of C mice with a colony bred from Andervont's animals.

It had been noted in previous work that implantation of pulmonary tissue or of pieces of the glandular part of the stomach along with methylcholanthrene led to a prompt squamous metaplasia and eventually to tumors of a transitional or frankly squamous sort (3, 4). With the new strain of mice, we tested these tissues against dibenzanthracene, 0.02 molar solution in olive oil, and obtained similar results. Figure 14 shows an adenoacanthoma induced from stomach tissue. It has been successfully propagated through 2 transfer generations, growing with great rapidity and killing new hosts within 6 weeks.

A group of 5 animals implanted with lung tissue merits special mention. Each had received the tissue merely with Locke's solution in one leg, in the other with 0.05 cc. of 0.02M dibenzanthracene in olive oil.

The first mouse was killed 113 days after implantation. At the control site there was a $4 \times 3 \times 1$ mm. patch of delicate, translucent tissue which the microscope showed to be alveolar lung tissue amidst which lay several structures resembling bronchi. The alveoli were collapsed. At the site where the tissue had been put with dibenzanthracene, the alveoli were distended, presenting the picture of adult lung. They must have contained fluid. This difference in the state of the alveoli at control and dibenzanthracene sites was true in the other mice of this series. It also occurs with methylcholanthrene (4). At the dibenzanthracene site, an adenoma 1 mm. across had formed amidst pulmonary alveolar tissue. A further change stood out, namely squamous metaplasia of bronchial epithelium. A cyst 3 mm. in diameter had been noted in the gross, and, when opened under water, a globule of oil floated out of it, establishing the fact that its lining must have been exposed to the dibenzanthracene contained in the oil. Microscopically, this cyst was found lined by tall columnar ciliated cells characteristic of bronchial epithelium. But these cells went over into a pseudostratified epithelium with 2 to 3 layers of cuboidal cells, and beyond these the cyst was lined by a frankly stratified epithelium composed of layers of flattened cells.

The next mouse, killed at 139 days, had a 12 mm. mass at the DBA site, sections of which are shown in Figures 15–19. A well defined adenoma lay in the midst of an area of lung tissue that elsewhere showed ten discrete mounds of squamous epithelial cells seated upon the walls of alveoli, a striking illustration of the ability of alveolar walls to undergo metaplasia. Two types of carcinoma had arisen in this specimen, one frankly squamous (Fig. 19), the other resembling adenocarcinoma (Fig. 18). On transplantation to new hosts, large tumors developed and sections showed them to be composed of both types of carcinoma seen in the original.

In animals killed at 190 and 213 days, sarcomas were found at the DBA sites, but had not completely destroyed the lung tissue, which showed the distended alveoli commented on above. The control implants in these mice resembled that described in the first animal.

The last mouse was killed at 250 days and bore a 3 mm. adenoma at the DBA site. Of greater interest was the finding of a large, histologically proven adenoma, 5 mm. in diameter, surrounded by a collar of lung tissue at the control site where the tissue had been implanted merely with Locke's solution. The significance of this finding will be commented upon in the discussion. We have seen "spontaneous" adenomas in the lungs of adult mice of this strain that have reached the age attained by this implant.

It is noteworthy that squamous metaplasia occurred not only in bronchial epithelium, but also in the walls of alveoli (Fig. 17), attesting to the existence of an epithelial lining in the alveoli (4).

DISCUSSION

When purified carcinogens were used, carcinomas appeared at practically every site where fragments of mouse embryo epidermis, suspended in 0.05 cc. of Locke's solution, were implanted with 0.05 cc. of 0.02 molar solutions of methylcholanthrene, benzpyrene, or dibenzanthracene. Cancers were elicited far more quickly with methylcholanthrene, being well established after 45 to 73 days in comparison to 73 to 175 days with dibenzanthracene or benzpyrene (Table 3). These differences may depend upon the conditions of test, for methylcholanthrene, like Scharlach R, possesses the property of attracting epithelial cells to migrate toward globules of oil containing it. It thereby comes to be quickly enclosed in the cysts that form at the implant sites, and the epithelial cells are thus selectively exposed to this carcinogen.

It was frequently noted at autopsy that oil containing dibenzanthracene, and sometimes oil containing benzpyrene, failed to become enclosed in the cysts. Incorporation of dibenzanthracene into the cysts could be assured by adding Scharlach R to the oil in which this carcinogen was dissolved. This procedure of itself did not speed the appearance of tumors. But with the concentration of dibenzanthracene reduced to a level that elicited tumors at only an occasional implant site, the addition of Scharlach R and croton oil increased the rate of appearance and the number of cancers obtained.

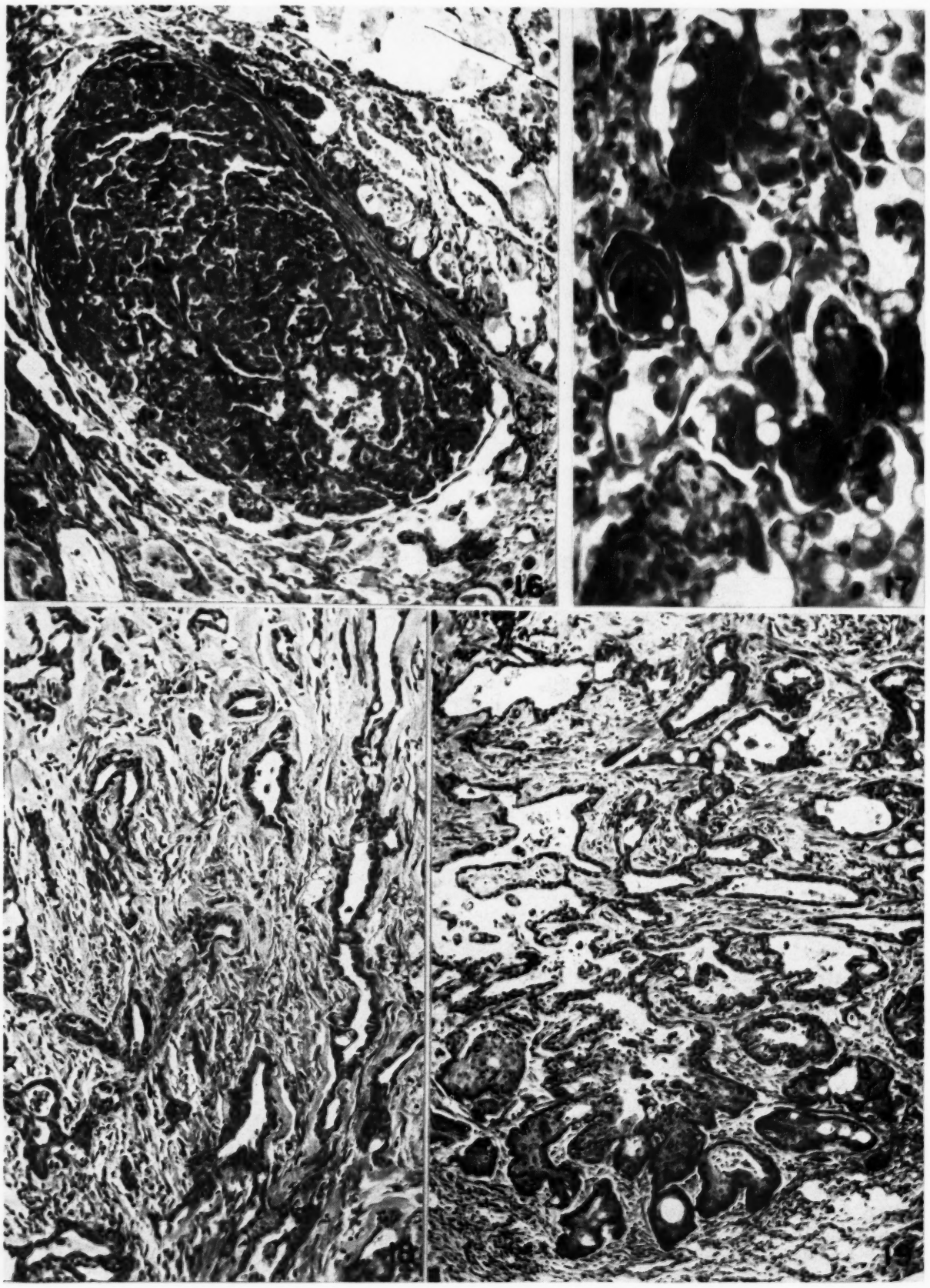


FIG. 16.—Higher magnification of the adenoma shown in Fig. 15. × 108.

FIG. 17.—Higher magnification of block B, Fig. 15, showing squamous metaplasia of alveolar walls. Note the discrete tufts of stratified squamous cells. × 245.

FIG. 18.—Detail of the growth of Fig. 15. In this area the tumor resembles adenocarcinoma. × 108.

FIG. 19.—Detail of the growth of Fig. 15. This type of growth, with cystic spaces and clefts lined by a few layers of flattened epithelial cells that desquamate without forming keratin, is commonly seen in tumors induced from lung, stomach or prostate tissue. The squamous nature of this tumor is apparent in the lower part of the photograph. × 108.

A considerable range of epithelial tissues and organs are amenable to test by this technic. Cancers have been easily and quickly induced from epidermis, gastric mucosa and pulmonary epithelium removed from mouse embryos and transplanted to adults together with methylcholanthrene (1 to 4, 8). In work as yet unpublished, we have elicited carcinomas from bladder and ureteral epithelium. Embryo tissues afford advantages because they can be obtained aseptically and grow well on transfer. In the case of certain organs, however, embryos yield too little tissue. In those instances, tissues from young adults, implanted together with crystals of methylcholanthrene, have served equally well, as Horning has shown with the prostate (10), Pan and Gardner with the uterine cervix (11). In our laboratory, a transplantable carcinoma has been induced from thyroid tissue taken from a young adult.

In our experience, the three carcinogens thus far discussed have caused a very prompt squamous metaplasia of pulmonary, gastric, thyroid, and prostate tissue, and the resulting tumors have been squamous or transitional cell cancers except in the case of lung tissue, which has yielded many adenomas. Adenomas appear within 3 to 4 weeks and are often multiple in grafts of lung tissue implanted with methylcholanthrene (4). In the present experiments, a large adenoma was found in a graft of lung tissue that had resided in a leg muscle for 250 days after implantation with Locke's solution. This is the only instance to date of the appearance of a tumor at the site of implantation of embryonic tissue without a carcinogen. But adenomas are not uncommon in the lungs of mice of this strain as they grow old (9), and it was not unexpected that some would appear in the transplanted lung tissue if the hosts were let live long enough. The present instance does not establish the point, for the control implant yielding the adenoma was in one leg of an animal that had received dibenzanthracene in its opposite leg. It is well known that subcutaneous injection of dibenzanthracene elicits adenomas in the lungs (9).

The tissue transplant technic requires only a single injection and is being investigated as a means for determining the carcinogenicity of materials that are available only in small quantities or which require considerable labor and expense to prepare in the bulk needed for test by painting, feeding or repeated injections. A carcinoma has been induced from skin tissue transplanted together with tar extracted from soot collected from the air in New York City. A papilloma has been obtained from gastric tissue transplanted with cottonseed oil that had been heated to 350° C. for 30 minutes. Chromatographic fractions of the tissues and urine of cancer patients and fractions of heated oils and fats are under examination.

SUMMARY

The tissue transplant technic affords a convenient means for assaying carcinogens directly against a variety of epithelial tissues.

The sensitivity of the test can be increased by addition of Scharlach R to solutions containing the carcinogen to insure incorporation of the carcinogen into the cysts formed by the implanted epithelium, and by addition of croton oil to increase the responsiveness of the tissue.

The pure substance, tricaprylin, can be advantageously used to replace olive oil as a solvent for this work.

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The Influence of the Degree of Caloric Restriction on the Formation of Skin Tumors and Hepatomas in Mice*

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Chronic caloric restriction inhibits the formation of various tumors of the mouse: spontaneous mammary carcinoma, spontaneous lung adenoma, induced skin tumor, induced sarcoma, and spontaneous and induced leucemia. Many workers have contributed to this knowledge, and the field has been recently reviewed (1). Studies have been conducted in an attempt to learn more about the conditions and factors that modify the caloric effect. Two of these (2, 3) have demonstrated that, for both the carcinogen-induced skin tumor and the spontaneous mammary carcinoma of the mouse, the extent of inhibition of tumor formation is directly dependent on the degree of caloric restriction.

The present paper is concerned with the extension of the studies on the effect of caloric restriction to the spontaneous hepatoma of the C3H male mouse. At the same time the relationship of graded caloric restriction to the formation of the carcinogen-induced skin tumor was re-investigated.

METHODS

Strain C3H male mice, bred in our laboratory, were utilized in these experiments. They were fed a commercial ration, Purina fox chow checkers, from weaning until the experimental diets were instituted. Litter mates were distributed among the groups of an experiment so far as possible. The mice fed *ad libitum* were housed 5 animals to a cage. In the groups fed calorie restricted rations, each set of 5 was kept in 2 cages; at biweekly weighings the lighter animals were placed in one cage, the heavier in the other. Throughout the experiment, the 5 calorie restricted mice of a set competed for the available food with others of nearly the same weight; thus they consumed approximately equal amounts of food.

TABLE 1

DAILY RATIONS EMPLOYED IN EXPERIMENT 1

Group	Purina fox chow meal (gm)	Skimmed milk powder (gm)	Cornstarch (gm)	Total Grams	Total Calories
1–23	1.4	0.9	0	2.3	8.1
1–26	1.4	0.9	0.3	2.6	9.2
1–29	1.4	0.9	0.6	2.9	10.3
1–32	1.4	0.9	0.9	3.2	11.4
1–36	1.4	0.9	1.3	3.6	12.8
1–40	1.4	0.9	1.7	4.0	14.3

The diets were composed of Purina fox chow meal, skimmed milk powder, and cornstarch. A week's supply of the weighed constituents was mixed with sufficient water to form an easily molded mash which was spread in pans, cut into blocks of appropriate size, and stored in a refrigerator (38° F). The diets were fed daily, usually at 1 P.M. All animals had free access to drinking water.

Each mouse was numbered and its course recorded separately. At 2-week intervals the animals were inspected for neoplasms and weighed. All mice were autopsied postmortem: when the tumors became large, at death, or at the termination of the experiment. Tumors were recognized grossly; microscopic sections were made of a considerable proportion of the lesions, as well as of the few that were questionable. Most of the carcinogen-induced skin tumors were papillomas when first observed and many of these progressed to carcinomas; a few were sarcomas. The hepatic tumors observed at autopsy were spontaneous benign hepatomas; they have been described previously by several investigators (4, 5, 6).

EXPERIMENTS

Experiment 1.—Six groups of 50 mice were employed. The daily rations for the mice of the several groups ranged from 2.3 to 4.0 gm. The ration for mice of group 1–23 was composed of 1.4 gm. Purina fox chow meal and 0.9 gm. skimmed milk powder. As indicated in Table 1, all diets con-

* This work was done through a Grant-in-Aid from the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council.

† Supported, in part, by the Michael Reese Research Foundation and the Foundation for Cancer Research, Chicago.

sisted of this basic ration to which varying amounts of cornstarch, from 0 to 1.7 gm., were added. Inasmuch as the cornstarch is almost entirely carbohydrate (and about 10 per cent water) the diets for the several groups contained the same amounts of protein, fats, vitamins, and minerals (contributed by the fox chow meal and the milk powder), and differed only in the amounts of carbohydrate. The computed caloric values ranged from 8.1 calories for the diet of group 1–23 to 14.3 calories for that of group 1–40. Correspondingly, as the amount of cornstarch was increased the proportion of protein and other essentials decreased.

The experimental diets were instituted when the mice were 8 to 10 weeks old. Initially, the mice of group 1–40 ate only 3.8 gm. of food, but within a few months they consumed all their daily ration. The mice of the other groups consumed their entire allotment of food from the outset. Three weeks after institution of the diets, the mice were given the first application of a 0.3 per cent solution of methylcholanthrene in acetone; a single drop of the solution was applied to the interscapular area by means of a dropping pipet. Seven such applications were given, at 5 day intervals, over a period of 30 days.

Changes in body weight of the mice are shown in Figure 1. Within 10 to 20 weeks after institution of the rations, the several groups reached average weight levels which were maintained fairly well throughout the experiment. These average body weight levels were approximately in direct proportion to the daily caloric intake. The experiment was terminated 51 weeks after the first application of carcinogen. The data relevant to formation of skin tumors are given in Table 2.

Groups 1–40, 36, 32, 29, 26, and 23 developed, respectively, 43, 32, 25, 22, 15, and 6 per cent skin tumors. Within the range of caloric intakes and at the dose of carcinogen employed, the inhibition of skin tumor formation was directly related to the degree of caloric restriction: The lower the caloric intake the lower the incidence of tumors, and in general, the longer the mean time of appearance of the tumors.

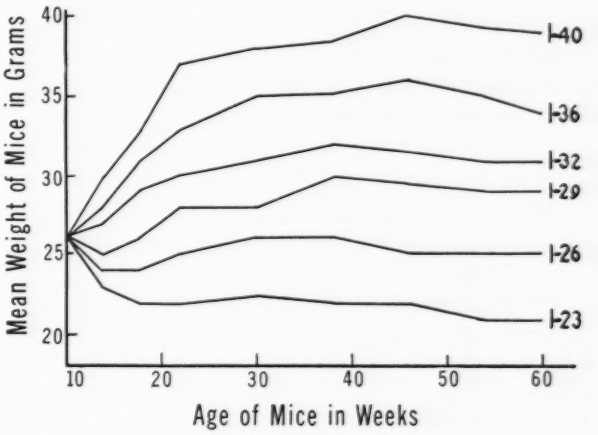

FIG. 1.—Growth of strain C3H male mice on different levels of caloric intake (Experiment 1).

TABLE 2

EFFECT OF LEVEL OF CALORIC INTAKE ON THE FORMATION OF SKIN TUMORS INDUCED BY METHYLCHOLANTHRENE

Group	Daily ration (gm)	Number* of mice	Week after first application of carcinogen: Per cent mice with skin tumors 10	20	30	40	final†	Mean time of appearance of skin tumors (weeks)	Number of mice alive and tumor-free at end of experiment†
1–23	2.3	37	0	0	0	6	6	39.0‡	30
1–26	2.6	45	0	2	2	9	15	36.2±5.7	33
1–29	2.9	50	4	4	10	12	22	32.5±4.7	38
1–32	3.2	48	8	8	10	17	25	29.0±4.9	34
1–36	3.6	50	12	16	18	22	32	25.5±4.6	32
1–40	4.0	49	14	19	25	31	43	25.5±3.9	27

* Initially there were 50 mice in each group. The "number of mice" is the total adjusted for deaths of mice without tumors (7).
† Experiment terminated 51 weeks after first application of carcinogen.
‡ The 2 tumors in this group were observed at the 39th week.

At the termination of the experiment the remaining animals were examined for hepatomas. A few of these mice had skin tumors; inasmuch as there was no correlation between the presence of a skin tumor and that of a hepatoma, all the mice, whether or not they had skin lesions, were employed for evaluating the incidence of hepatomas. The pertinent data are given in Table 3. Groups 1–40, 36, 32, 29, 26, and 23 had, respectively, 64, 47, 15, 11, 0, and 0 per cent heptaomas. As with the induced skin tumor, the incidence of hepatomas among the several groups decreased with decreasing caloric intake. The decrease in mean size of the hepatomas with decrease in caloric intake suggests that the hepatomas may have originated later in the more restricted groups. However, the differences in size are not large and might be de-

pendent on modification of the growth rates by the different caloric consumptions.

Experiment 2.—In the preceding experiment caloric restriction was achieved by limiting only the amount of carbohydrate; this means of caloric restriction leads to a greater proportion of protein, fat, vitamins, and minerals in the rations of the restricted animals. The present experiment was undertaken to ascertain whether formation of the spontaneous hepatoma, like other mouse tumors, responds to simple underfeeding (all components of the ration reduced proportionately) in the same manner as to caloric restriction *per se* (restriction in carbohydrate only).

TABLE 3

EFFECT OF LEVEL OF CALORIC INTAKE ON INCIDENCE OF SPONTANEOUS HEPATOMAS

Group	Daily ration (gm)	Number* of mice	Per cent with hepatomas	Size of hepatomas (mm) Range	Mean
1–23	2.3	32	0		
1–26	2.6	38	0		
1–29	2.9	46	11	3–11	6.0±1.5
1–32	3.2	39	15	2–16	5.2±2.2
1–36	3.6	38	47	2–17	8.0±1.2
1–40	4.0	36	64	2–20	9.4±1.2

* Including mice bearing skin tumors; see text. Mice 62 to 64 weeks old.

TABLE 4

EFFECT OF UNDERFEEDING ON THE INCIDENCE OF SPONTANEOUS HEPATOMAS

Group	Daily ration (gm)	Number* of mice	Per cent with hepatomas	Size of hepatomas (mm) Range	Mean
n42	2.5	21	0		
n40	4.0	50	44	2–24	6.9±1.3

* Mice 58 to 59 weeks old.

Two groups of mice, 13 to 14 weeks of age, were employed. The diet was composed of Purina fox chow meal, 40 per cent, skimmed milk powder, 20 per cent, and cornstarch, 40 per cent. The mice of group n40 (50 animals) were given 4.0 gm. daily, those of group n42 (29 animals) 2.5 gm. On the average, the mice of group n40, ate from 3.8 to 4.0 gm. and subsequent to 7 months of age weighed between 37 and 40 gm. The mice of group n42 ate the 2.5 gm. given them and, after 4 months of age, weighed between 22 and 24 gm., on the average.

When the mice were 58 to 59 weeks of age, they were sacrificed and examined for hepatomas. The data are given in Table 4. Forty-four per cent of the mice of group n40 had hepatomas; no hepatomas were observed in the 21 mice of group n42 surviving to the end of the experiment. In agreement with the results of Experiment 1, a 35 to 40 per cent reduction in food intake significantly inhibited the formation of hepatomas.

DISCUSSION

Inhibition of formation of spontaneous hepatomas in the C3H male mouse by chronic caloric restriction was achieved either by limiting only the carbohydrate (caloric restriction *per se*) or by proportionate reduction of all components of the diet. This finding is in accordance with similar observations for all previously studied tumors of the mouse. It was also shown that the incidence of hepatomas was dependent on the degree of caloric restriction. The results on the formation of skin tumors, obtained here with a moderate dose of carcinogen, agrees with previously published experiments in which higher doses of carcinogen were utilized (2): With decreasing caloric intake below the *ad libitum* level, there is a decreasing incidence of tumors, or a delay in their appearance, or both.

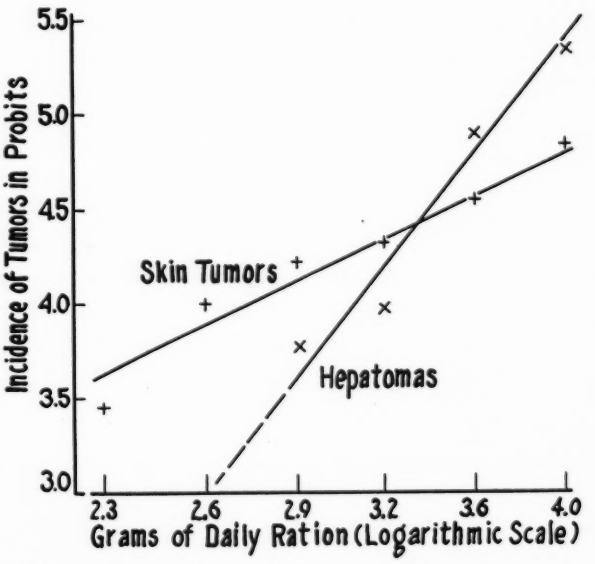

FIG. 2.—Linear relationship between incidence of tumors in probits (Y) and the logarithm of the daily food intake (X). For hepatomas, $Y = 12.6\,X - 2.20$; for skin tumors, $Y = 4.92\,X + 1.85$. As tested by the chi-square method, the straight lines satisfactorily fit the data, the respective chi-square values being 5.1 and 0.85.

The data may be examined by methods analogous to those employed in biological assay.[1] When the probit of the incidence of spontaneous hepatomas is plotted against the logarithm of the daily food intake, a very nearly linear relation is obtained (Figure 2). A similar linear dependence ex-

[1] The terminology and statistical techniques applicable to the discussion are presented by Fisher and Yates (8). Probits are the transformation of the percentage incidence to areas of the normal distribution curve, these areas being measured in standard deviation units.

ists for the induced skin tumor (Figure 2). The greater slope of the regression line for hepatomas, as compared with that for skin tumors, quantitates the greater response of hepatoma formation to caloric restriction.

Previously published data on the formation of both spontaneous mammary carcinoma and benzpyrene-induced skin tumors in mice (2, 3) were analyzed in the same way and, within the limits of statistical requirements, the probits of the incidence of tumors were also linear functions of the logarithms of the caloric intakes. Referred to units of per cent animals with tumors, plotted against caloric intake on an arithmetic scale, the data imply that when the tumor incidences range between 0 and 100 per cent, the graph of the relation between tumor incidence and caloric intake should be a $\int$ shaped curve with the upper arm longer than the lower arm—i e. an integration of a positively skewed distribution curve.

It was previously suggested that there might be a critical level at which tumor inhibition is most readily affected by a unit change in caloric intake. Now it is evident that the point of maximum change in tumor incidence is not dependent on a particular level of caloric intake, but rather is determined by the range of tumor incidences between the highest and lowest levels of food intake. Inasmuch as the probit of the incidence of tumors is a linear function of the logarithm of the food intake, the greatest change in the percentage of tumors, for proportionate changes in food intake, occurs in the region of a 50 per cent incidence of tumors. This is supported by the present and previously reported (2, 3) graded caloric restriction experiments, in which the tumor incidences in the *ad libitum* group to the most restricted group ranged a) from 100 to 0 per cent; b) from 100 to near 50 per cent; and c) from near 50 to 0 per cent, as obtained in Experiment 1 with skin tumors and hepatomas.

SUMMARY

1. Chronic caloric restriction inhibits the formation of the spontaneous hepatoma of the C3H male mouse. This caloric effect is in accord with that obtained with all previously studied tumors.

2. In an experiment in which graded caloric intakes were given to several groups of mice, it was demonstrated that with decreasing caloric intake there was a decreasing incidence of both methylcholanthrene-induced skin tumors and spontaneous hepatomas.

3. The relationship between caloric intake and incidence of these two types of tumors may be mathematically stated: the probit of the incidence of tumors is a straight line function of the logarithm of the daily caloric intake. This implies, and the data agree, that the largest inhibition of tumor formation, for proportionate reduction in food intake, occurs near the level of 50 per cent incidence of tumors.

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Heterologous Transplantation of the Brown-Pearce Tumor*

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The extent of the field open to study in an investigation of the constitutional relationships of cancer is greatly expanded by heterologous transplantation. It becomes possible, for example, to compare the behavior of a human mammary carcinoma in normal mice and in mice bearing spontaneous breast tumors or, on the other hand, to study the course of a mouse cancer in such diverse constitutional types as the guinea pig and the hamster. A variety of studies of this nature have been undertaken in this laboratory and one of the tumors employed has been the Brown-Pearce carcinoma. The Brown-Pearce carcinoma is an epidermoid carcinoma of the scrotal skin of a rabbit and has been used extensively in cancer research, but, as far as can be determined from the literature, the tumor has not been transferred or studied outside of the parent species. Consistent variations in behavior occur in other animal species and their possible relationship to known differences in constitution is the subject of continued investigation. Later papers will be concerned with such aspects of the study but the fact that the tumor is transplantable heterologously is not generally recognized, and because of its significance from an economical as well as a scientific point of view this warrants present emphasis.

MATERIALS AND METHODS

The Brown-Pearce tumor is maintained in the rabbit by serial anterior chamber transfer. At this site 100 per cent of takes are obtained and it is not necessary to use more than one animal in each succeeding generation. Growth is rapid and the chamber is regularly filled by the seventh day in the winter and by the tenth day in the summer. Metastasis occurs in from 4 to 6 weeks during cold weather but regression without metastasis is the rule during the hot months. Growths obtained in the anterior chamber from 7 to 10 days after transfer formed the source of tumor tissues used in the heterologous experiments to be described.

Mice, rats, hamsters and guinea pigs were utilized as heterologous hosts. The mice included representatives of the C3H, dba, Bagg albino, A albino, C57 black, C57 brown, and Swiss strains. The pure strain mice were bred in our laboratory and the Swiss were purchased from a commercial house. The rats were of the Rockland albino strain; the guinea pigs and hamsters were run-of-the-mill stock supplied by local breeders. The animals were maintained under ordinary conditions of cage life and received no special treatment. Neither age nor sex appeared to influence the results of transfer.

The anterior chamber of the eye, the subcutaneous and intramuscular spaces, the testicle and the kidney were used as transplantation sites. The anterior chamber technique has been described in detail (1, 2). Subcutaneous transfers were made into the axillary region and the thigh muscles were used for intramuscular transplantation. Testicular and renal transfers were effected by means of a 20 gauge hypodermic needle fitted with a plunger. After immobilization of the organ with the fingers and incision of the overlying skin, the needle was thrust into the substance of the organ and the tumor fragment expelled. Testicular transplantation is easily executed but some practice is required before equal proficiency is attained in kidney transfers. Tissue fragments rather than cellular emulsions were used in all cases and surgical sterility was maintained in all operative procedures.

In some instances transfers were made in association with embryonic tissues. Embryos were obtained at different stages of development and the desired organ dissected. The embryonic organ and the tumor tissue were then loaded into the trocar and expelled together in the selected region.

RESULTS

The Brown-Pearce tumor was readily transplanted to mice, rats, and hamsters, but all attempts to transfer to guinea pigs failed.

An important difference between the Brown-Pearce tumor and other tumors studied in relation to heterologous transfer was noted early in the course of the investigation. In the case of other tumors, it had been found that a preliminary transfer to the anterior chamber of the new species with subsequent use of the eye-grown tumor was

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essential to successful transfer to different bodily regions of other individuals. It is significant from several points of view that this does not hold in the case of the Brown-Pearce tumor and immediate transfer of tissue from the rabbit's eye to the subcutaneous space or testicle of the mouse or hamster is followed by takes and growth.

Transfer to Mice.—Approximately 100 per cent of takes occur on transfer of the Brown-Pearce tumor to the anterior chambers of mice, irrespective of strain, but in other bodily locations, the incidence varies in relation to this factor.

Eye.—Growth in the anterior chamber is apparent within two days of transfer and when the transplantation procedure has been carried out with care, there is no evidence of inflammatory reaction. The transplanted fragment grows as a whole and the focal growth with residual regression characteristic of many tumor transplants rarely occurs.

The chamber is consistently filled with tumor by the fourth or fifth day but the subsequent course of the transplant varies (Fig. 1). If the operative incision at the corneo-scleral junction is not fully healed, the wound ruptures and the growing tumor protrudes as a fungating mass. This is rapidly followed by infection and the ensuing inflammatory reaction destroys the tumor and the eyeball. The rapidity of healing in the corneal wound depends largely on the amount of care exercised at operation. A torn or triangular incision such as results from the use of a dull or single edged knife is usually followed by herniation of the iris and delayed healing. In like manner, too large an incision or one involving the sclera rather than the cornea, retards healing and may lead to rupture with infection and destruction of the tumor. With proper technique, healing is sufficiently advanced by the fifth day so that rupture of the wound does not occur and the growing tumor produces a uniform bulging of the cornea.

Complete regression may occur at this stage and in such instances, the animals are resistant to further inoculation. More frequently regression is incomplete and after the bulk of the tumor has disappeared, new foci of proliferation appear and these in turn may grow to fill the chamber. Recurrence is usually rapid but may be delayed for a long period of time. In a number of cases, renewed growth was not evident until after the lapse of six months. The process of regression and recurrence may be repeated a number of times but eventually no further growth occurs and on test the animal is found to be resistant to further transfer of the tumor.

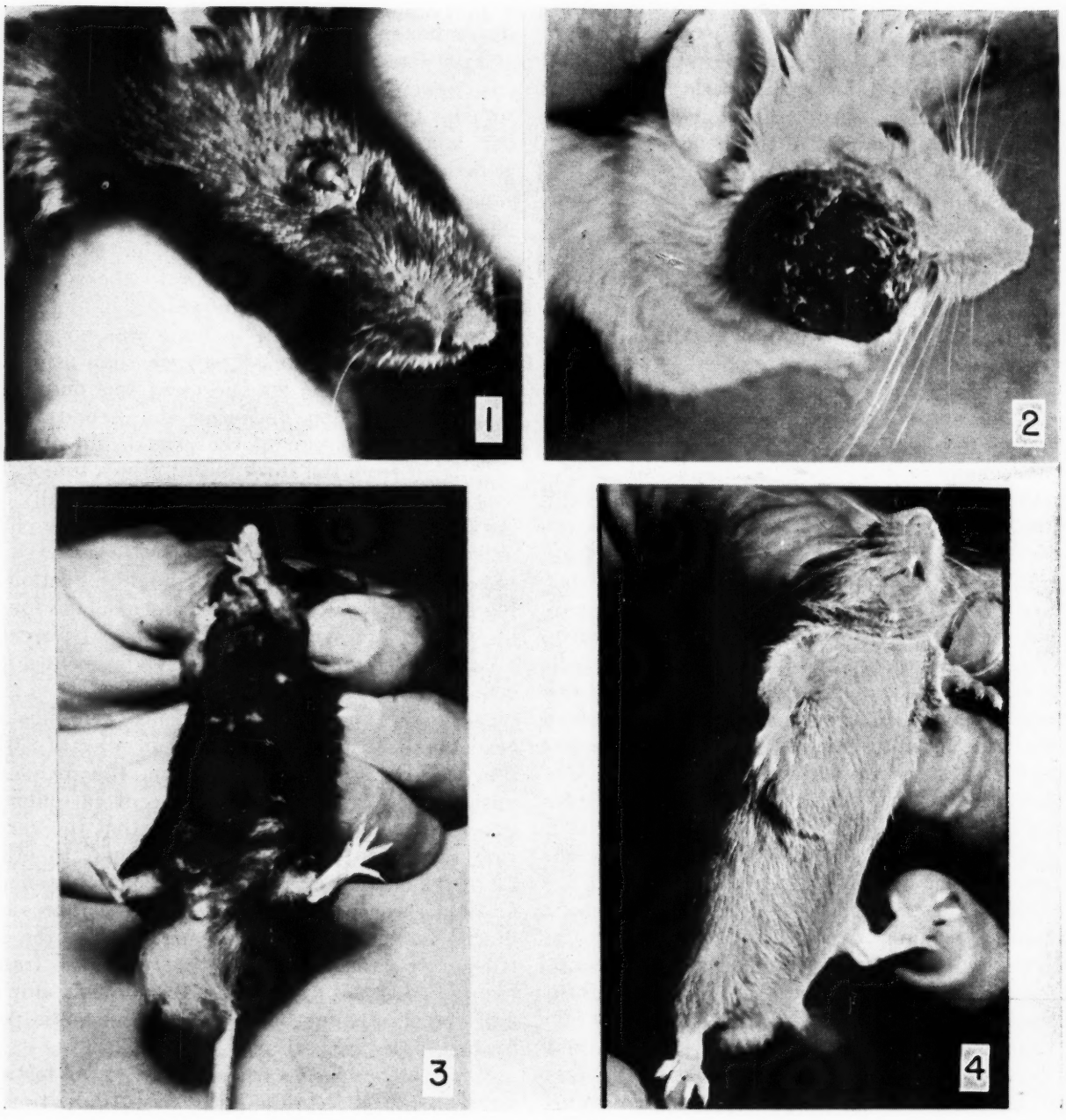
In other cases, the cornea is destroyed by the expanding tumor and the fungating mass protrudes externally (Fig. 2). Growth may continue for several months until eventually the animal dies of infection or complete regression occurs. In no instance has metastasis been observed.

Transfer to a new generation may be made at any time prior to complete regression. Routine transfer has been performed on the tenth day in our laboratory and by continued passage the growth has been carried through more than 30 serial generations. At various times throughout mouse passage, the tumor was returned to the rabbit and, as is usually the case, on the return of a heterologously grown tumor to the species of origin, both growth rate and rapidity of metastasis were increased, but the increase was no greater after the thirtieth mouse passage than after the first. Moreover, the enhancement was only temporary in duration, and after the second rabbit passage the behavior of the growth did not distinguish it from the stock rabbit grown tumor.

The tumor was not altered histologically by growth in the mouse and showed the same characteristics after the thirtieth passage as serve to identify it in the rabbit (Figs. 5 and 6). Section of regressing tumors showed the same changes found at similar stages in rabbits with broad irregular areas of necrosis and intact surviving cells adjacent to blood vessels.

Testicle.—The highest incidence of takes on testicular transfer was obtained in mice of C3H, dba and Swiss strains. In these, the incidence varied between 50 and 100 per cent in different generations and it seems probable that the variation relates to technique, rather than to other factors. The mouse's testicle is considerably smaller than the rabbit's and any undue compression during the course of transfer may lead to the extrusion of parenchyma together with the transplanted fragment. The procedure is much simpler and the results far better when transfer is performed under general anesthesia.

Growth is apparent on palpation of the testicle between the fifth and seventh days. Palpation is sometimes difficult due to withdrawal of the testicle into the abdomen but with slight persistent pressure the organ can be forced back into the scrotum. The enlargement is sufficiently great by the tenth or twelfth day that the testicle can no longer pass through the inguinal ring and occasionally it is found trapped in the abdomen. The enlargement is uniform rather than nodular on palpation and continues until a mass 1 to 2 cm. in diameter is reached (Fig. 3). The edema associated with growth in the rabbit's testicle is minimal and "metastatic orchitis" is extremely rare.



FIGS. 1–4

FIG. 1.—C57 mouse bearing anterior chamber transplant of Brown-Pearce tumor. Photograph was taken 5 days after transfer.

FIG. 2.—Bagg albino mouse bearing anterior chamber transplant of Brown-Pearce tumor. Photograph was taken 50 days after transfer.

FIG. 3.—C57 mouse bearing testicular transfer of the Brown-Pearce tumor. Photograph was taken 20 days after transfer.

FIG. 4.—Mouse of dba strain bearing subcutaneous transplant below right axilla. Photograph was taken 17 days after transfer.

The duration of growth is variable but eventually growth terminates in regression rather than in metastasis. Regression may occur shortly after a take is noted or be delayed for a month or more. Recurrence of growth such as occurs in the eye has not been observed in the testicle, and, following regression, the animals are resistant to re-inoculation. Serial transfer is readily carried out and the tumor can be maintained indefinitely by continued passage.

Mitoses are numerous and the cells appear and are arranged exactly as in the rabbit (Fig. 7). Testicular parenchyma is destroyed and replaced and the tumor may extend along tubules to involve the epididymis. Neither lymphatic extension nor metastasis has been observed.

Very large growths, as much as 2.5 cm. in diameter, have been found in intra-abdominal testicles and in this situation, intact, living cells may persist for two months or longer. In order to supply more material for a study of such growths, a series of experiments was carried out in which the tumor bearing testicle was immobilized in the abdomen at laporatomy. The duration and extent of growth were increased but invasion of other organs did not occur and regression was the eventual outcome in all cases.

Kidney.—The enhanced growth noted in the above cases suggested an attempt to grow the tumor in intraabdominal organs. Takes were sometimes obtained in the liver and occasionally growing fragments of tumor were found attached only to peritoneal surfaces but by far the best results were obtained on transfer to the kidney. The technique employed was a "hit or miss" affair and only about a quarter of the animals used showed evidence of growth at autopsy.

Palpation through the abdominal wall is unsatisfactory in early stages of growth but a definite enlargement can be felt in successful cases by the twelfth day. Growth may persist for a month or more, giving rise to an irregular mass in the flank readily seen in the living animal. In several instances, the tumor has broken through the kidney capsule and invaded the liver and adrenal, but in these as in all other cases, the eventual outcome is regression.

On section, wide areas of kidney substance are replaced by tumor showing the same characteristics observed in other bodily regions (Fig. 8). The method of transfer is traumatic and results in the production of extensive scars and it is of some interest that these as well as the renal parenchyma are involved in spread of the tumor.

The tumor can be carried by serial kidney passage but the difficulties involved render the procedure impractical except for special undertakings. Routine transfer for stock tumor supply is more easily accomplished by utilizing the eye or the testicle but, as will be noted below, the ideal transplantation site for such purposes is the subcutaneous space.

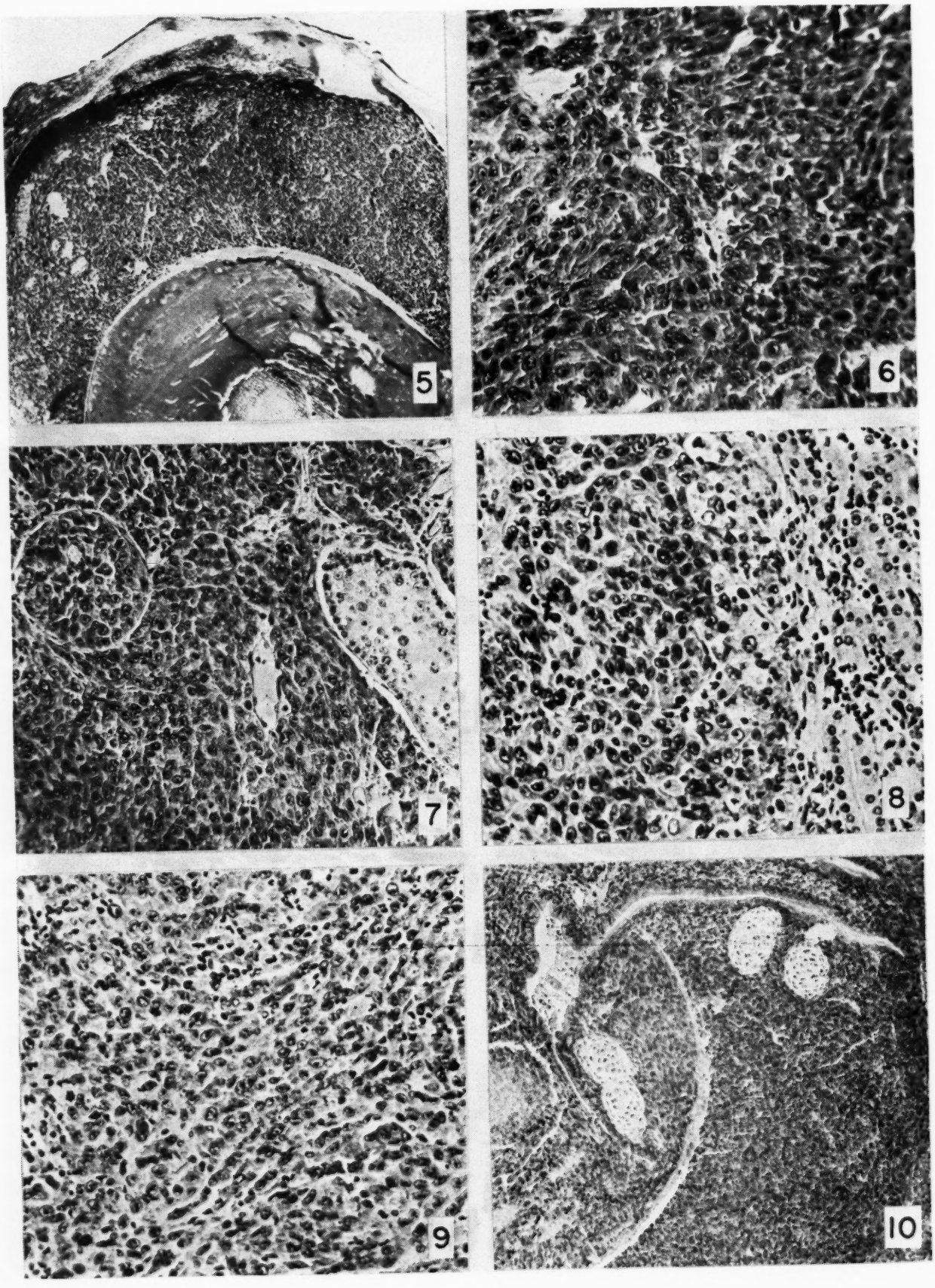
Subcutaneous Space.—Transfer to the subcutaneous space is readily effected in mice of dba and C57 brown strains. The dba's support larger growths than the browns and this strain has been used for routine experimental purposes. Fewer takes occur in other strains and the resulting growths never attain an equal size.

The presence of growing tumor can be determined by palpation on the fifth day, and the growth increases to form a mass from 1 to 2.5 cm. in diameter during the ensuing 2 weeks (Fig. 4). Intact, viable cells may persist for as long as 2 months but eventually, as in other locations, the tumor undergoes necrosis. Serial passage can be made at earlier periods and the tumor maintained indefinitely by continued subcutaneous transfer.

It should be emphasized that transfer is made directly from the rabbit's eye to the subcutaneous space of the mouse without an interposed transfer to the mouse eye. This is unusual in our experience with heterologous transplantation and the question arose as to whether or not the ability to grow in extra-ocular locations in the mouse was a characteristic of other rabbit cancers. Unfortunately, only one other rabbit cancer was available for test. This was an epidermoid carcinoma of the skin induced with carcinogenic chemicals by Dr. Francisco Duran-Reynals. It had been carried for several generations in the rabbit's anterior chamber in our laboratory and the eye-grown tumor was used for subcutaneous transfer in the mouse. As in the case of the Brown-Pearce carcinoma, takes occurred and the tumor can be carried in the mouse by serial subcutaneous transfer. The finding is suggestive and other rabbit cancers will be tested as they become available.

Histologically, subcutaneous transplants of the Brown-Pearce carcinoma are identical with the parent tumor (Fig. 9). Regressive changes are first noted in areas remote from blood supply, and cells in the vicinity of blood vessels survive longest. The mass of necrotic tumor persists for a considerable period of time after regression and is eventually organized into a fibrous scar.

Embryonic Organs.—Transfer of the tumor in association with mouse embryonic parts gives rise to takes in all strains tested. Any type of embryonic organ or tissue can be used and any transplantation site elected. In strains such as the C3H or the C57 black in which the growth of tumor tissue



FIGS. 5–10

alone rarely exceeds a matchhead in size, the tumor-embryonic organ combination may attain a diameter of a centimeter or more. On histological examination, however, the tumor is found to be sharply limited to the embryonic part and does not extend into the tissues of the adult host. Further, the growth in the embryonic tissues is invasive in character and not expansive as in the tissues of normal adult animals (Fig. 10). The significance of this observation lies in the fact that it contrasts embryonic tissue and normal adult tissue and relates embryonic tissues and the adult tissues of a spontaneous tumor bearing host, for in the latter case, growth is also invasive.

Spontaneous Tumor Bearing Mice.—The results of transfer to C3H mice bearing spontaneous autonomous mammary tumors are in sharp contrast to those obtained in normal C3H mice. In normal C3H mice, takes are rare and the resulting growth seldom exceeds a quarter of a centimeter in diameter. On the contrary, 100 per cent of takes and large tumors measuring 2 cm. in diameter may be obtained in C3H mice with spontaneous mammary cancers. It should be emphasized that the enhanced growth of the transplants does not occur as long as the mammary tumors remain dependent and is observed only in mice whose spontaneous tumors have attained autonomy as manifested by heterotransplantability.

In the latter animals growth is rapid and subcutaneous transplants invade the skin and underlying muscle. In several cases, sections obtained from the lung at autopsy have shown nests of cells resembling the Brown-Pearce tumor, but whether they represent metastasis of the transplants or anaplastic metastasis of the spontaneous breast cancer has not been determined.

Transfer to Rats and Hamsters.—The results of transfer to rats and hamsters were not materially different from those observed in mice. Good growths were obtained in the eyes and testicles of both species but subcutaneous transfers were generally more successful in hamsters than in rats (Figs. 11–14). Growth in all sites ultimately terminated in regression and the animals were resistant to re-inoculation.

Transfer to Guinea Pigs.—Numerous attempts were made to transfer the tumor to guinea pigs using the anterior chamber, testicle, and subcutaneous space as transplantation sites and tissue derived from rabbits, mice, hamsters, and rats as inoculum. All such attempts failed and in no instance was there any indication whatsoever of growth.

In view of the successful transplantation to mouse embryonic tissues, an effort was made to transfer the tumor to guinea pigs' eyes in association with guinea pig embryonic organs. The transplanted material grew well to fill the anterior chamber in from 10 to 15 days, and at this time representative pigs were killed for examination. One half of the contents of the chamber was saved for section and the remainder was transferred to rabbits' eyes. The results were consistently the same. The sectioned tissue consisted entirely of growing embryonic tissue and was completely devoid of Brown-Pearce tumor cells. Yet, after a latent period of several weeks, growth occurred in the rabbits' eyes and on histological examination proved to be the Brown-Pearce tumor. It would appear, therefore, that isolated viable tumor cells were present in the transplants and were unrecognized despite careful study. Confirmatory evidence that such was the case was provided in two instances in which serial section of transplants 25 days after transfer showed small foci of intact tumor cells. The tumor cells apparently do not survive beyond this period for at 30 days and after, neither serial section nor rabbit transfer give evidence of their presence.

DISCUSSION

The object of the present paper has been to report the heterotransplantability of the Brown-Pearce carcinoma. It should be emphasized that this property is not peculiar to the Brown-Pearce tumor but, in our experience, is shared by all growths capable of invasion and metastasis. Justi-

FIG. 5.—Section of eye of C3H mouse bearing transplant of Brown-Pearce tumor. Section was taken 8 days after transfer and shows the tumor filling the expanded anterior chamber. Mag. ×40.

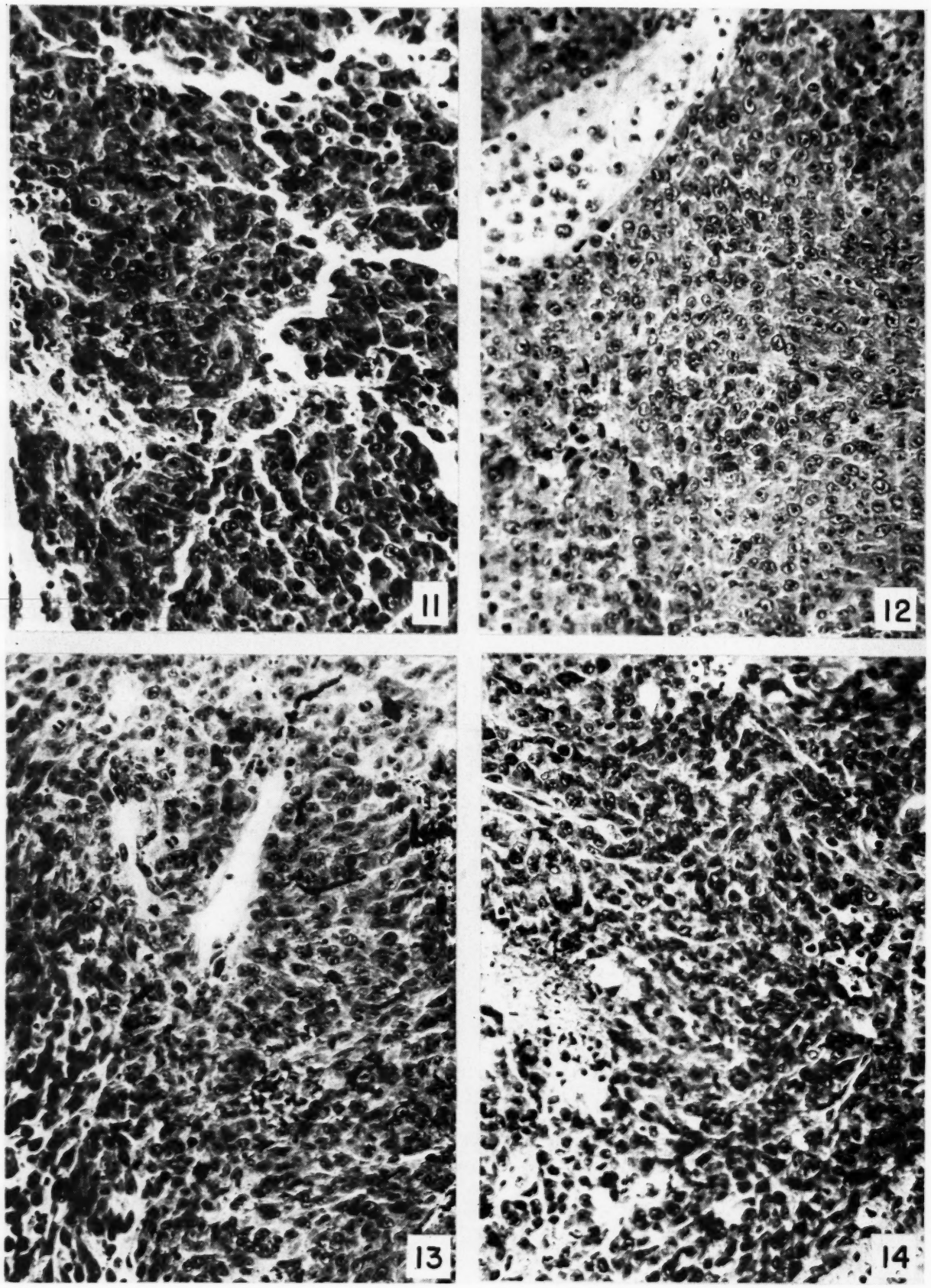
FIG. 6.—Higher power view of tumor in previous section showing Brown-Pearce cells in typical arrangement. Mag. ×300.

FIG. 7.—Section of testicle of Swiss mouse bearing transplant of Brown-Pearce tumor. A single intact tubule remains; elsewhere testicular parenchyma is destroyed. Near the top of picture is a tubule with intact capsule filled with tumor illustrating one mode of spread. Mag. ×215.

FIG. 8.—Section of kidney of C3H mouse 20 days after transfer of the Brown-Pearce tumor. Renal parenchyma is visible on one side of the photograph and tumor on the other. Mag. ×300.

FIG. 9.—Section of subcutaneous transplant of Brown-Pearce tumor in a dba mouse 16 days after transfer. Mag. ×300.

FIG. 10.—Section of Brown-Pearce tumor growing in a subcutaneous transplant of embryonic lung in a C57 black mouse. The lung parenchyma has been completely destroyed by tumor growth and only small areas of bronchial epithelium and cartilage remain. Mag. ×90.



FIGS. 11–14

FIG. 11.—Section of Brown-Pearce tumor in a rat's eye 14 days after transfer. Mag. ×300.

FIG. 12.—Section of Brown-Pearce tumor in a rat's testicle 22 days after transfer. Mag. ×300.

FIG. 13.—Section of Brown-Pearce tumor in a hamster's eye 20 days after transfer. Black cells are pigmented cells of the iris invading the transplant. Mag. ×300.

FIG. 14.—Section of subcutaneous transplant of Brown-Pearce tumor in a hamster 11 days after transfer. Mag. ×300.

fication of special consideration of the Brown-Pearce in this respect lies in its economical bearing on research with this tumor and no attempt has been made in the present note to go beyond a purely descriptive account of the salient facts of heterologous transfer.

Variations in the behavior of the tumor in different species and constitutional types are of greater scientific interest and future reports will be based on an investigation of their significance. The point of concern in these studies is not the success of heterologous transplantation but rather its failure in certain circumstances and the unusual behavior of transplants in others. The known constitutional differences in the respective hosts suggest a causal relationship and form the basis of an experimental approach.

The sharply defined range of transplantability with inclusion of the mouse, hamster, and rat, and exclusion of the guinea pig, parallels the pattern of vitamin C metabolism in these species. A similar relationship has been noted in the case of other tumors, that is, a grouping of species with reference to the ability to synthesize vitamin C coincides with susceptibility or resistance to transfer. Transplantation between species with the same type of C metabolism (man and guinea pig) is comparatively easy while transfer between species with different types (man and rabbit) is difficult. The possible influence of this factor on the results of transfer is open to experiment and pertinent investigation is in progress.

The contrasting fate of transplants in C3H mice bearing spontaneous cancers and in normal animals of the same strain, further emphasizes the position of constitutional factors in tumor behavior. Few takes occur in normal C3H mice; growth is purely expansive and rapidly terminates in regression. On the contrary, transplants grow readily and to large size in C3H mice bearing autonomous primary tumors. Moreover, their tissues allow invasion and, although evidence is based only on morphological similarity, it seems probable that metastasis occurs. Comparable variations in behavior have been observed on transfer of other tumors to such animals. A mouse sarcoma (MT-8), transplanted to normal C3H mice grows expansively for a full month before invasion occurs and gross metastasis can rarely be found before the sixth week. In contrast, invasive growth begins immediately on transfer to C3H mice with autonomous spontaneous mammary tumors and metastases are commonly found as early as the eighteenth day.

The phenomena of invasion and metastases are uniquely characteristic of cancer and represent the most striking departure from normal biological behavior associated with the disease. It is generally believed that they arise from developments in the tumor alone and the concept that their actuation may be conditioned by constitutional factors is not widely held. The results obtained with the Brown-Pearce and other tumors are highly suggestive and experiments designed to give further data are under way. It is of considerable interest in this connection that the reaction between the Brown-Pearce tumor and embryonic tissue are comparable with that observed in the normal tissues of mice bearing spontaneous cancers.

SUMMARY

The heterologous transplantation of the Brown-Pearce tumor has been described. The tumor was successfully transplanted to the eyes, testicles and subcutaneous tissues of mice, hamsters and rats, but failed to grow at any site in guinea pigs.

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The Action of Oxazine Dyes on Tumors in Mice[1]

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The discovery that oral administration of the oxazine dye, Nile blue A, to tumor-bearing mice resulted in diffuse staining of the tumor cells and significant retardation of their growth (1) led to the investigation of all available oxazine dyes. Further reports (2, 3) describe the action of a few of these dyes not only on transplanted sarcomata, but also on primary induced sarcomata and spontaneous mammary gland carcinomata.[2] The present report summarizes the data previously reported together with new data obtained from a number of oxazine dyes synthesized by M. L. Crossley and associates[3] for the study of the correlation between the structural nature of the compounds and their action on malignant growths.

MATERIAL AND METHOD

The compounds were administered by mixing them into the food given to the animals. The details of this procedure, the handling of the animals and the method of implantation of the tumors are described in previous papers. Four inbred strains of mice, the A, B.A., C3H and C57, and two strains of rats, the pedigreed Wistar rat and an inbred strain of Wistar rats, were used.

Fifty-five dyes were tested as shown in Table 1. The system of nomenclature and numbering is that employed by Chemical Abstracts. The Colour Index[4] numbers of the commercially available phenoxazine dyes tested were 877 to 886; 888; 892 to 894; 896; 900 and 905. The commercial benzo[a]phenoxazines tested were 909; 913; 914; 915 and cresyl violet.[5]

The dyes were fed in 0.2 to 0.4 per cent concentrations, depending upon their toxicity.

Feeding of the dyes was begun on the day the tumors were implanted and the mice were sacrificed 14 or 15 days later. The majority of the untreated mice bearing tumors died at about 16 days. At this time they had tumors that measured in the neighborhood of $30 \times 20 \times 16$ mm. or about 9,600 cu. mm.

The tumor was considered as retarded in growth if its volume was less than 1000 cu mm. at the time of sacrifice. The presence of dye in the urine or in the bile of treated mice was used as evidence that it was absorbed from the alimentary tract. Staining of tumor was judged by its color (deep blue, blue, or pale blue) on visual examination.

RESULTS

A tabulation of the dyes used, the occurrence of staining, and the retardation of tumors in mice fed the dyes is given in Table 1. Seventeen of the dyes were phenoxazines and 38 were benzo[a]-phenoxazines. Forty-three mice were fed phenoxazine dyes and 526 mice were fed benzo[a]phenoxazine dyes.

Phenoxazines.—No member of this group stained tumors although some of them caused retardation of tumor growth. Since none of these dyes stained tumors, they are not listed individually in Table 1, but are grouped according to the general nature of the substituting group. The presence of the dyes in the bile or urine indicated that these dyes, with the exception of the 3,8-diamino-7-keto-phenoxazines, were absorbed from the intestines. Two of the dyes, brilliant cresyl blue (2-methyl-3-diethylamino-7-amino-phenoxazine), and gallocyanin (3-diethylamino-6-hydroxy-9-carboxylic acid-7-amino-phenoxazine), caused staining of the bladder epithelium. In such instances color appeared in specific granules characteristic of the large epithelial cells covering the inner surface and not as a diffuse staining of the entire cell.

[1] Aided by a grant from the National Cancer Institute, Bethesda, Maryland.

[2] Samples of commercially available dyes were generously provided for our studies by the various manufacturers listed in our previous publications. In addition, a number were synthesized especially for these studies.

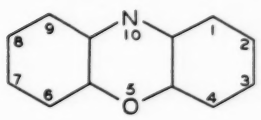
[3] The Calco Chemical Division, American Cyanamid Company.

[4] The Colour Index, published by the Society of Dyers and Colourists, London, England, 1924.

[5] Cresyl violet, an oxazine dye, produced by the National Aniline Division, Allied Chemical & Dye Corp., has not been given a Colour Index number.

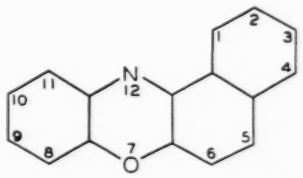
TABLE 1

PHENOXAZINES



	No. of mice	No. of tumors retarded	No. of tumors stained
3-aminophenoxazine	9	0	0
3-amino-7-keto-phenoxazine (oxazone)	16	0	0
3,7-diaminophenoxazine	8	3	0
3,8-diamino-7-keto-phenoxazine (oxazone)	8	2	0
3,9-diamino-7-keto-phenoxazine (oxazone)	2	0	0

BENZO[A]PHENOXAZINES



9-amino derivatives			
A. 9-dimethylamino-benzo[a]phenazoxonium salt (Meldola's blue—Colour Index No 909)	21	7	0
B. 9-diethylamino-benzo[a]phenazoxonium chloride	15	6	0
" " " base	14	6	0
C. 9-diethylamino-11-methyl-benzo[a]phenazoxonium chloride	5	5	0
D. 9-diethylamino-2-hydroxy-benzo[a]phenazoxonium salt (Muscarine—Colour Index No. 915)	11	1	0
9-amino-5-keto-derivatives			
A. 9-diethylamino-5-keto-benzo[a]phenoxazine (Oxazone of Nile blue A)	7		7
B. 9-dimethylamino-10-methyl-5-keto-benzo[a]phenoxazine (Oxazone of cresyl violet)	3	1	3
5,9-diamino derivatives			
A. 5-amino-9-diethylamino-benzo[a]phenazoxonium salts (Nile blue A)	157	125	149
" " " —base	4	4	4
B. 5-amino-9-dimethylamino-10-methyl-benzo[a]phenazoxonium salt (Cresyl violet)	5	3	5
C. 5-allylamino-9-diethylamino-benzo[a]phenazoxonium chloride	8	8	8
D. 9-diethylamino-5-ethylamino-benzo[a]phenazoxonium salt	8	6	6
E. 5,9-bis(diethylamino)-benzo[a]phenazoxonium salt	6	4	0
F. 5-acetamino-9-diethylamino-benzo[a]phenazoxonium salt	10	10	0
G. 5-benzylamino-9-diethylamino-benzo[a]phenazoxonium chloride (Nile blue 2B)	58	29	58
9-diethylamino-5-(4′-methylbenzylamino)-benzo[a]phenazoxonium chloride	22	15	22
9-diethylamino-5-(4′-methoxybenzylamino)-benzo[a]phenazoxonium chloride	9	6	9
9-diethylamino-5-(3′,4′-methylenedioxybenzylamino)-benzo[a]phenazoxonium chloride	10	7	10
9-diethylamino-5-(2′,3′-dimethoxybenzylamino)-benzo[a]phenazoxonium chloride	4	4	4
9-diethylamino-5-(4′-nitrobenzylamino)-benzo[a]phenazoxonium chloride	8	0	0
5-(4′-carboxybenzylamino)-9-diethylamino-benzo[a]phenazoxonium chloride	14	7	0
5-(4′-bromobenzylamino)-9-diethylamino-benzo[a]phenazoxonium chloride	5	4	5
5-(4′-chlorobenzylamino)-9-diethylamino-benzo[a]phenazoxonium chloride	12	10	12
5-(2′-chlorobenzylamino)-9-diethylamino-benzo[a]phenazoxonium chloride	3	2	0
9-diethylamino-5-(4′-iodobenzylamino)-benzo[a]phenazoxonium chloride	6	6	6
H. 9-dimethylamino-5-phenylamino-benzo[a]phenazoxonium chloride	7	0	0
5-(4′-chlorophenylamino)-9-dimethylamino-benzo[a]phenazoxonium chloride	4	2	0
I. 9-diethylamino-5-phenylamino-benzo[a]phenazoxonium chloride	16	8	16
5-(4′-chlorophenylamino)-9-diethylamino-benzo[a]phenazoxonium chloride	4	1	0
9-diethylamino-5-(4′-hydroxyphenylamino)-benzo[a]phenazoxonium chloride	6	0	0
9-diethylamino-5-(3′-hydroxyphenylamino)-benzo[a]phenazoxonium chloride	4	4	4
9-diethylamino-5-(2′-phenylphenylamino)-benzo[a]phenazoxonium chloride	6	0	0
J. 9-diethylamino-5-(1′-naphthylamino)-benzo[a]phenazoxonium chloride	3	0	0
K. 9-dimethylamino-2-hydroxy-5-phenylamino-benzo[a]phenazoxonium salt (Fast Green M—Colour Index No. 916)	3	1	0
L. 9-dimethylamino-5-(2′-pyridylamino)-benzo[a]phenazoxonium chloride	3	0	0
M. 9-diethylamino-5-(2′-pyridylamino)-benzo[a]phenazoxonium chloride	10	8	0
N. 9-diethylamino-5-(2′-thiazolylamino)-benzo[a]phenazoxonium chloride	9	2	0
O. 9-diethylamino-5-(2′-pyrimidylamino)-benzo[a]phenazoxonium chloride	16	7	0
P. 5-[2′(3″-bromopyrrolidyl)]amino-9-diethylamino-benzo[a]phenazoxonium chloride	6	2	3
Q. 9-diethylamino-5-(4′-acetaminobenzensulfonamido)-benzo[a]phenazoxonium salt	4	0	0

Benzo[a]phenoxazines.—Eighteen different benzo[a]phenoxazine dyes stained the tumors in mice to which they were fed. They were diamino- or monoamino-monoketo-benzo[a]phenoxazines. With the exception of the keto compounds, they all contained amino or substituted amino groups in both the 5- and 9-positions. Four compounds tested in which a 5-amino group was not present did not stain tumors. No compound in which the 9-amino group was absent was available for testing.

The greatest number of compounds synthesized so far have aralkyl substituents in the 5-amino group. This synthetic procedure was followed because it was found that a benzyl derivative of Nile blue A, namely commercial Nile blue BB,[6] was superior to Nile blue A.

Varying the nature of the substituents in the amino groups of the 5- and 9-position resulted in compounds which showed differences in toxicity, tumor staining, and degree of selectivity as judged by coloration. Mono-substitution in the 5-amino group with the biphenyl, naphthyl, pyridyl, pyrimidyl, and thiazolyl radicals interfered with staining. From our limited data it appeared that mono-substitution in the 5-amino group with the benzyl radical resulted in a dye (Nile blue 2B) which was less toxic than Nile blue A dyes in which the 5-amino group was unsubstituted. The lesser toxicity was shown in the longer survival of normal mice fed Nile blue 2B as against those fed on similar percentages of Nile blue A. The majority of the benzo[a]phenoxazine dyes were excreted in the bile as well as the urine. This led to various amounts of coloration of the liver due to the passage of the dye through its cells. The different Nile blue A dyes tested varied in amounts of staining and retardation of growth of tumors in mice. They all stained tumor tissue less deeply than Nile blue 2B. All samples of Nile blue 2B dyes brought about intense staining of sarcomata and carcinomata (both the primary and the transplanted malignant growths). The dye, 9-diethylamino-5-(4′-methoxybenzylamino)-benzo[a]phenazoxonium chloride, stained tumor tissue a deep blue, but it also stained various organs as well as the connective tissue of mice to which it was fed.

DISCUSSION

In our earlier reports it was shown that the dyes that stained tumor tissue were naphthindone BB (monoazo-azine); ethyl violet (triphenylmethane); acridine orange (acridine); Nile blue A, Nile blue 2B and cresyl violet (oxazines); 3-dimethylamino-7-(2′diethylaminoethylamino) phenazthionium iodide (thiazine); 1,4-diamino-anthraquinone and 1,4,5,8-tetramino-anthraquinone (anthraquinones).

In general there seemed to be some relationship between diffuse staining of tumors and the retardation of their rate of growth. In a study of the action on tumor-bearing mice of acridine compounds (4), it was found that the majority of the acridine compounds that stained the tumors retarded their growth. In the present study of oxazine dyes, the results show that the majority of these dyes that stained tumors also brought about retardation of their growth. Riley (5), who measured the tumors in a number of mice which were given diets containing Nile blue and neutral red dyes, also found that the tumors which were stained were smaller than those not stained.

It has been shown (3) that the Nile blue A dyes prepared by different manufacturers (Calco, Ciba, Clayton, Geigy, Grübler and National Aniline) brought about different degrees of staining and retardation of tumors in mice. Even different batches of the same dye manufactured by the same company gave different results, as, for instance, the Nile blue A made by National Aniline many years ago stained sarcomata and carcinomata blue and caused marked retardation of tumor growth while the Nile blue A manufactured by them today stained tumors pale blue and brought about only slight retardation of growth.

Even though growth of tumors was greatly retarded in the mice fed certain dyes, complete prevention or regression of growth did not occur. Premedication with Nile blue A and Nile blue 2B dyes had no significant deterrent effect upon the growth of subsequent tumor grafts.

In earlier studies (2, 3) it has been shown that the action of Nile blue differed in the various species of animals to which it was administered. The difference between mice and rats was especially marked. Nile blue A and Nile blue 2B dyes failed to give consistent results in regard to staining or retardation of tumors in rats. Mixing caronamide[7] or sodium benzoate with the dye, added to the diet, improved staining in some instances.

It has been shown previously that tumors in rats and mice become stained following injections of certain acridine and xanthene dyes, but not when solutions of Nile blue were injected. The failure to obtain staining in animals injected with

[6] Nile blue BB is the proprietary name of the National Aniline Division for their Nile blue 2B. Nile blue BB, C.I. #914, is a quite different dye from Nile blue A, C.I. #913, made by the same company. Nile blue BB, like all Nile blue 2B dyes, stains tumor tissue deep blue, while Nile blue A (N.A.D.) stains tumor pale blue.

[7] Supplied by Sharp & Dohme, Inc.

solutions of Nile blue A or 2B was due to the local deposition of the dye at the injection site when water or saline was used as a solvent. However, when a 0.2 per cent solution of the dye in 5 per cent glucose was injected it remained soluble and caused the staining of tumors in both rats and mice within 1 to 3 hours after subcutaneous or intraperitoneal injection.

Atrophy of the thymus and some coloration of the thymus, lymph nodes, and testicles occurred in mice that received dyes that stain and retard tumor growth. In animals fed with acridine compounds, not only were the liver cells colored by the passage of the dye through them during excretion in the bile, but the granules of the liver cells themselves were also stained. This action which probably caused some damage to the liver was interesting in connection with studies on tumor-bearing rats fed mixtures of dyes. In rats fed quinacrine for a short time previous to, or coincident with, Nile blue 2B, considerable blue as well as yellow was taken up by the tumors and they became bright green. The percentage of yellow to blue in the green color depended upon the concentration and length of time each dye was administered.

The staining of the large specific granules present in the epithelial cells covering the inner surface of the urinary bladder that took place in mice fed with brilliant cresyl blue and gallocyanin was also noted in mice that were fed certain thiazine dyes, namely methylene blue, methylene green, toluidine blue, new methylene blue HSG and new methylene blue N, Colour Index numbers 922, 924, 925, 926 and 927 respectively (3).

Riley (5) studied the action of dyes on tumor growth in mice. He found that in addition to the dyes previously reported to stain tumors, the azine dye, neutral red, C.I. #825, also stained and retarded tumor growth *in vivo*. Riley used Nile blue (Gurr), Nile blue sulphate (source unknown) and Nile blue chloride (British Drug Houses, Ltd.) of which we had no samples. He states that the feeding of Nile blue A to mice caused *in vivo* staining and inhibition of growth of carcinoma, but to a much less extent when sarcoma 180 was employed. The formula 3-diethylamino-naphthophenoxazine hydrochloride, which is recorded as "hydrochloride of Nile blue Base," prepared by the British Drug Houses, Ltd., is a misprint.[8] In our experiments and also those of Riley, 3-diethylamino-naphthophenoxazine (the diethylamino homologue of Meldola's blue) did not stain tumor tissue.

Allam, Goland and Parkins (6) found that normal and tumor-bearing dogs were able to take Nile blue 2B (both N.A.D. and Calco) orally in doses of 0.3 gram per kilogram per day for long periods of time. Nile blue 2B dyes proved to be less toxic than Nile blue A. The vivid gross coloration of tumors obtained in mice was not seen in dogs.

Bates and Kershman used Nile blue 2B (BB of N.A.D.) on some recent investigations on brain tumors (7). They found that orally administered dye stained induced and transplanted intracerebral sarcoma, carcinoma, and glioma in mice. The neoplastic tissue was diffusely stained blue while the surrounding brain remained unstained. Their experiments in mice, dogs, and one patient led them to think that this dye might be used in efforts to stain human brain tumors.

Sloviter (8) has prepared the halogenated derivatives of Nile blue 2B and also the radioactive iodo derivative. The use of the radioactive dye is under investigation.

The mechanism involved in selective staining of tumors is still obscure. Certain tiny granules in the cortex of the adrenal gland become stained a deep blue in mice fed Nile blue 2B. These granules reduce silver nitrate in acid solution and have been shown to be ascorbic acid (9). It is well known that tumor tissue has a greater content of ascorbic acid than normal tissue. We have been unable to show a relationship between ascorbic acid and Nile blue staining in tumor since the ascorbic acid present in the tumor does not take the form of tiny blue granules as it does in the adrenal cortex of mice fed Nile blue, nor does the ascorbic acid in the tumor reduce silver in the form of tiny black granules when it is fixed in acidified silver nitrate.

SUMMARY

Fifty-five different oxazine dyes were administered orally to tumor-bearing mice to compare the structural features of these compounds in relation to their ability to produce diffuse staining of tumor tissue. Of these compounds, 17 were phenoxazines, 38 were benzo[a]phenoxazines.

Eighteen structurally different benzo[a]phenoxazines stained and retarded tumors in mice to which they were fed. There was some relationship between diffuse staining and retardation of tumor growth. Seventeen structurally different phenoxazines failed to stain tumors in mice to which they were fed, but a few caused some retardation of tumor growth.

With the exception of the benzo[a]phenoxazines which contain a carbonyl (keto) group in the 5-position, all compounds that stained tumor tissue had amino or substituted amino groups in both the 5- and 9-position. The four compounds tested in which a 5-amino group was not present did not

[8] Personal communication from Dr. Riley.

stain tumor tissue. No compound was tested in which the 9-amino group was absent. Varying the nature of the substituted amino groups in the 5- and 9-positions resulted in compounds which showed marked differences in toxicity, tumor staining, and in degree of selectivity (as determined by coloration alone).

Mono-substitution in the 5-amino group with the benzyl radical produced a dye (Nile blue 2B) which stained tumor tissue more intensely and was less toxic than Nile blue A dyes in which the 5-amino group is unsubstituted. The lesser toxicity was shown in the longer survival of normal mice fed Nile blue 2B, as against those fed on similar percentages of Nile blue A.

Dyes produced by mono-substitution in the 5-amino group with alkyl, alkenyl, aryl and aralkyl radicals stained tumor tissue with but few exceptions. Similar substitution by three heterocylic radicals interfered with staining.

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The Stimulating Effect of Dietary Fat on Carcinogenesis*

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The stimulating effect of fat on the rate of formation of certain types of tumors is well established (18, 19, 22), yet no complete explanation for this phenomenon has been offered (1, 2, 13, 15, 16, 19, 22 to 24). Evidence is presented in this report which indicates that the difference in the net energy value of low and high fat diets is sufficient to explain the stimulating effect of fat on the induction of skin tumors with carcinogenic hydrocarbons.

METHODS

Female mice of the "Rockland strain" 2 to 3 months of age at the start of the experiment were used. The animals were kept in hanging type screen bottom cages. Each group consisted of 48 animals divided among 3 or more cages. Water was allowed *ad libitum* from bottles. Every morning the rations were weighed into feeders especially designed for this purpose (20) with the aid of a Toledo balance sensitive to 0.1 gm. The diets were mixed at intervals of 1 to 3 weeks and were stored in a refrigerator.

Tumors were produced by the application twice weekly of 1 drop of a solution of 0.3 gm. of benzpyrene in 100 ml. of benzene to the shaved skin of the interscapular area. The dropper delivered about 20 μl. of benzene per drop containing 60 μg. of benzpyrene. The mice were weighed and inspected for tumors and physical condition at weekly intervals. The numbers of mice showing papillomas and carcinomas were recorded separately and mice with carcinomas were removed from the experiment. Diagnosis was verified on representative tumors by microscopic examination. The incidence of tumors was calculated on the basis of the number of mice showing carcinomas and it was expressed as per cent of the effectual total (the number of mice alive in each group on the day the first carcinoma was observed in the experiment).

The composition of all rations (presented in detail in Table 1) was based on that of the ration designed for *ad libitum* consumption, which is shown in the first column of Table 1. The content of glucose in this ration could be reduced to give a desired restriction in calories as shown in the second, third, and fifth diets, or a selected amount of fat could be substituted isocalorically for glucose (fourth, fifth and sixth diets). Thus, the mice ingested the same amount of protein, minerals, vitamins, and unsaturated fat source irrespective of alterations in ration composition and level of caloric intake.

EXPERIMENTS AND RESULTS

Experiment 1. A comparison of tumor incidence in mice fed low and medium fat diets at different levels of caloric intake.—The mice were divided into 6 groups of 48 mice each. One group was fed the high calorie-low fat diet *ad libitum* and food consumption was recorded. These records showed that the mice consumed an average of 3.45 gm. of ration per mouse per day over the entire experimental period. This was equivalent to 12.1 calories per mouse daily. Another group was also fed the high calorie-low fat diet, but at a level of 10 calories (2.84 gm.) per mouse each day. A third and fourth group were restricted respectively to 8 calories of the medium calorie-low fat diet and to 6 calories of the low calorie-low fat diet (2.26 gm. and 1.67 gm.) per mouse each day. The medium fat-high calorie diet was fed to the mice of a fifth group at a level of 10 calories (2.0 gm.) per mouse each day, and the medium fat-low calorie diet was offered to the last group at 6 calories (1.17 gm.) per mouse daily. These rations were fed for 26 days before the first of the 38 semiweekly applications of benzpyrene was made.

The growth response of the mice to the 4 levels of caloric intake was in agreement with that re-

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TABLE 1

COMPOSITION OF DIETS

Diet	1 High Calorie Low Fat			2. Medium Calorie-Low Fat			3. Low Calorie-Low Fat			4. High Calorie-Medium Fat			5. Low Calorie-Medium Fat			6. High Calorie-High Fat		
Constituent	Per cent	Parts	Cal./100 gm. of diet	Per cent	Parts	Cal./100 gm. of diet	Per cent	Parts	Cal./100 gm. of diet	Per cent	Parts	Cal./100 gm. of diet	Per cent	Parts	Cal./100 gm. of diet	Per cent	Parts	Cal./100 gm. of diet
Cerelose[1]	78	78	265.2	72.3	57.3	245.8	62.5	36.6	212.2	44.2	31.0	150	22.4	9.3	76.2			
Primex[2]										24.4	17.2	227	24.2	10.0	225.0	56.5	28.5	526.0
Casein[3]	15	15	66.0	18.9	15	83.2	25.6	15	112.8	21.4	15.	94	36.4	15	160.0	29.7	15.	130.8
Corn oil	2	2	18.6	2.5	2	23.2	3.4	2	31.6	2.8	2	26	4.9	2	45.6	4.0	2	37.2
Liver concentrate[4]	1	1	2.0	1.3	1	2.6	1.7	1	3.4	1.4	1	2.8	2.4	1	4.8	2.0	1	4.0
Salt mixture[5]	4	4		5.0	4		6.8	4		5.7	4		9.7	4		7.9	4	
Total	100	100	351.8	100.0	79.3	354.8	100.0	58.6	360.0	99.9	70.2	499.8	100.0	41.3	511.6	100.1	50.5	698.0
Grams per mouse each day and the caloric equivalent[6]	2.84 gm., 10.0 cal.			2.26 gm., 8.0 cal.			1.67 gm., 6.0 cal.			2.0 gm., 10.0 cal.			1.17 gm., 6.0 cal.			1.43 gm., 10.0 cal.		

The following amounts of B vitamins were added to each 15 gm. of casein used in all diets (mg.): thiamine 0.3, riboflavin 0.3, pyridoxine 0.3, nicotinamide 0.5, calcium pantothenate 2.0, biotin 0.01, pteroylglutamic acid 0.1, p-aminobenzoic acid 100, inositol 100, and choline 200.

Halibut liver oil to furnish 21 units of vitamin A and 0.3 units of vitamin D per mouse each day was added to the corn oil (6 drops HLO per 20 gm. of corn oil).

[1] Cerelose—a pure glucose monohydrate.

[2] Primex—a partially hydrogenated vegetable oil.

[3] Ordinary commercial dietary casein.

[4] Liver powder, 1:20 concentrate. The Wilson Laboratories, Chicago.

[5] Phillips, P. H., and Hart, E. B., Jour. Biol. Chem., **109**: 657, 1935.

[6] The figures are given as an example only. In practice, the *ad libitum* level of consumption of the high calorie diet determines the amounts of the other diets offered as follows: the number of grams of a ration allowed each mouse per day must be in the ratio of the total number of "parts" of that diet; ie., 100:79.3:58.6:70.2:41.3:50.5 respectively as listed in the table. This will result in a daily caloric intake per mouse on each of the six diets, expressed as per cent of the first diet, of 100, 80, 60, 100, 60 and 100% respectively.

Calories were calculated on the following basis: (see Bodansky, M. Introduction to Physiological Chemistry, p. 510, 4th ed. New York: John Wiley and Sons, Inc., 1938).

1 gm. casein	4.4 cal.
1 gm. cerelose	3.4 cal. (91 per cent glucose)
1 gm. fat	9.3 cal.
1 gm. liver conc.	2.0 cal.

ported by Tannenbaum (25). Mice fed diets in which fat was substituted isocalorically for glucose were heavier, an observation which has been made by others (5, 8, 14). These data are summarized in Table 2. Deaths from non-tumor causes were low in all groups except those fed the low fat diet *ad libitum* and the medium fat diet at the high calorie level.

fat content of the diet was increased from 2 to 27 per cent. The data on tumor incidence are given in Table 3 and the relationship between caloric intake and tumor incidence is plotted in Fig. 1.

Experiment 2. A comparison of the tumor incidence in mice fed a low fat diet and a high fat diet free of carbohydrate, at the same calorie level.—A group of 48 mice was fed the high calorie-low fat diet

TABLE 2

WEIGHTS OF MICE IN EXPERIMENT 1

	Low Fat Diet					Medium Fat Diet				
	Days on Diet					Days on Diet				
Caloric intake Cal./mouse/day	1	42	97	139	181	1	42	97	139	181
	Wt. in gms.					Wt. in gms.				
12.1	24.5	31.2	34.0	33.5	34.7					
10.0	24.4	30.4	31.6	31.2	31.9	24.5	29.7	31.6	31.7	32.9
8.0	24.6	26.6	25.4	25.8	25.6					
6.0	24.3	22.3	21.0	20.8	20.4	24.9	23.8	21.6	21.8	21.5

TABLE 3

THE TUMOR INCIDENCE OF MICE IN EXPERIMENT 1

		Cumulative Tumor Count (173 Days)			
		Number of Mice			
Calorie intake Cal./mouse/day	Effectual total (71 days)	Alive and tumor free	Papilloma	Carcinoma	Per cent carcinoma
Low fat diet					
12.1	39	3	2	32	82
10.0	44	4	7	28	64
8.0	47	9	8	23	49
6.0	44	19	13	8	18
Medium fat diet					
10.0	47	3	10	34	72
6.0	42	7	14	14	33

The incidence of tumors varied directly with the caloric intake in the 4 groups fed the low fat diets, ranging from 82 per cent in the group which consumed 12.1 calories per mouse daily to 18 per cent in the group restricted to 6 calories per mouse

TABLE 4

WEIGHT RECORD SUMMARY OF MICE FED ISOCALORIC AMOUNTS OF THE 2 PER CENT AND OF THE 61 PER CENT FAT DIETS

	Days on Diet			
Diet	1	60	123	194
	Weight in Grams			
2% fat	25.2	25.2	27.7	27.4
61% fat	24.9	26.5	28.0	27.9

daily. Furthermore, the isocaloric substitution of fat for glucose resulted in an increased tumor incidence. Thus at the 10 calorie level the tumor incidence was 64 per cent in the group fed the low fat diet and 72 per cent in the group fed the diet containing 27 per cent of fat, and mice restricted to 6 calories per day showed an increase in tumor incidence from 18 per cent to 33 per cent when the

and a comparable group was fed the high calorie-high fat diet which was free of carbohydrate. The composition of the diet is given in Table 1. Both groups were fed at a level which furnished 9.5 calories (2.7 gm. and 1.36 gm., respectively) per mouse each day. This was about 10 per cent below the *ad libitum* intake of these mice. After the experimental diets had been fed for 40 days, the first of the 38 semiweekly applications of 1 drop of 0.3 per cent methylcholanthrene[1] in benzene was applied to the shaved interscapular area of each mouse.

The gross appearance, survival, and the condition at autopsy of the mice fed the 61 per cent fat diet was similar to that of the control mice. Both groups consumed their daily rations within 8 hours after they were offered. As shown in Table 4, the average gain in weight of the mice fed the high fat-carbohydrate free diet was about the same as that of mice fed the more conventional diet. The final

[1] Benzpyrene was not available at the time of the experiment.

incidence of carcinomas was 64 per cent in the control group and 78 per cent in the group fed the 61 per cent fat diet, a 22 per cent greater incidence (Table 5). The experiment was repeated using benzpyrene as the carcinogen, and the high fat diet promoted a higher incidence of tumors by 29 per cent.

DISCUSSION

The mechanism of the action of dietary fat in stimulating carcinogenesis has been under investigation for some time (1, 2, 13, 15, 16, 19, 22 to 24, 27). At present it is recognized that even with caloric intake and local effects controlled there is a residual effect of fat which tends to augment the formation of certain tumors (19, 22). One explanation for this effect is that fat contains a factor that acts as a procarcinogen in stimulating tumor formation. If this were true, the augmenting effect should be proportional to the amount of fat in the diet. However, in these experiments this did not prove to be the case; there was no greater stimulation of tumor formation with 61 per cent of fat in a carbohydrate free ration than with 27 per cent of fat. It is unlikely, therefore, that there is any direct stimulating action of fat *per se*, or of a metabolite of fat, acting on the developing tumor cell.

Rusch, Kline, and Baumann (19) pointed out that this residual effect of fat may be concerned with the efficiency of utilization of food energy. The first experiment was carried out to test this suggestion. Evidence has accumulated since 1944 (3, 6 to 12, 21) that shows a decreasing energy expense of utilization (or a decreasing specific dynamic action) of diets fed in isocaloric quantities in the order of their increasing fat content. The curve shown in Fig. 2 illustrating this point was plotted from an average of the data of Forbes, *et al.* (9, 11). At the 2 per cent level of fat the energy expense of utilization of the diet averaged about 34 per cent of the gross caloric intake. This would amount to about 3.4 calories out of 10 calories for the low fat diet. Similarly, it can be shown that the dynamic effect of a 27 per cent fat diet would average about 19.5 per cent of the gross caloric intake, or 2.0 calories per 10 calories. Thus the net energy of a 27 per cent fat diet fed at the level of 10 calories would be greater than that of a 2 per cent diet by the difference between 2.0 and 3.4 calories, or about 1.4 calories per mouse each day.

The first experiment was designed to permit interpretation of the results on the basis of the data of Forbes, *et al.* (Fig. 2). Thus in Fig. 1 the curve shows the relation between caloric intake and tumor incidence of mice fed the 2 per cent fat diet. The incidence of cancer is also indicated for mice fed the 27 per cent fat diet at the levels of 6 and 10 calories. It is apparent from the figure that the tumor incidence of mice fed the 27 per cent fat diet at the 10 calorie level is of the same order of magnitude as that which would result from 11 calories of the 2 per cent fat diet. Therefore, the greater net energy value of the 27 per cent fat diet, which was shown in the preceding paragraph to be

TABLE 5

TUMOR INCIDENCE OF MICE FED ISOCALORIC AMOUNTS OF THE 2 PER CENT AND OF THE 61 PER CENT FAT DIETS

		Cumulative Tumor Count (214 Days)			
		Number of Mice			
Diet	Effectual total (116 days)	Alive and tumor free	Papilloma	Carcinoma	Per cent carcinoma
2% fat	39	4	5	25	64
61% fat	46	4	4	36	78

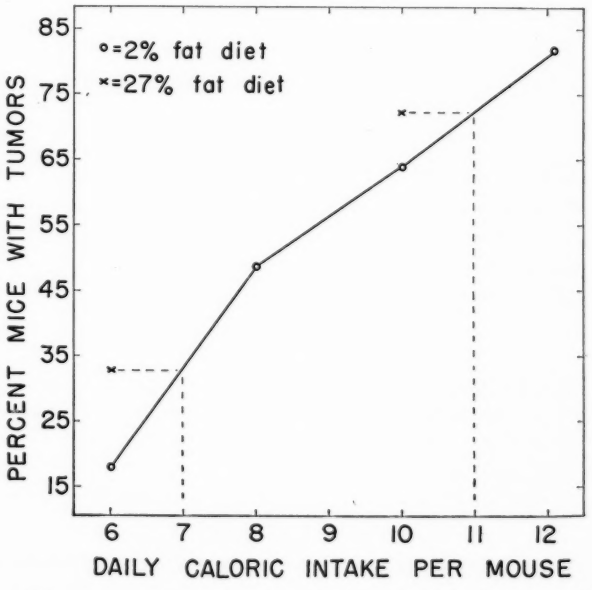

FIG. 1.—A curve showing the relationship between the daily caloric intake of mice and the incidence of tumors.

about 1.4 calories, is sufficient to account for all the unexplained procarcinogenic action of fat. The same conclusion can be reached by similar calculations in the case of the mice fed the 6 calorie diets at the two levels of fat. It is concluded that the major effect of dietary fat on certain types of carcinogenesis is indirect, mediated through an increase in the net energy available to the animal.

Although the question arises concerning the application of values obtained with rats to the interpretation of data determined with mice, the principle of energy expended in the utilization of food is general among all mammals tested (4). It is probable that greater variation in the net energy

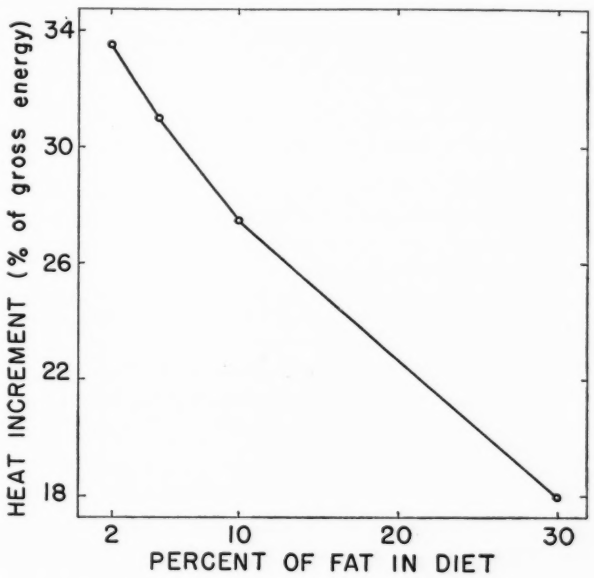

FIG. 2.—A curve showing the relationship between the energy expense of utilization and the fat content of a diet (Data taken from Forbes, *et al.* (9, 11)).

value of diets may arise from differences in the use to which the ingested nutrients are put and in many other circumstances than from species differences.

Tannenbaum reported (26) that increments of fat in the diet had a greater effect on tumor formation in the range of 2 to 4 per cent total fat content than in the range of 4 to 16 per cent of fat. These results correlate with the finding of Forbes, *et al.* (8) and of Swift and Black (21) that there is a greater differential in efficiency of utilization if fat is increased from 2 per cent to 5 per cent (34 calories greater net energy value) than if it is increased from 5 per cent to 30 per cent (20 calories greater). However, the tumor incidence of mice fed the 61 per cent fat diet cannot be correlated with its net energy value, since the energy expense of utilization of this diet is unknown.

Within recent years investigators have become aware of the importance of maintaining isocaloric levels of food consumption in experimental and control groups of animals when comparing the influence of various factors on carcinogenesis. It now appears that rations that differ in their content of fat, carbohydrate, or protein must be fed at equivalent net energy levels before a unique effect of the experimental changes on tumor formation can be established. These findings also raise the question of the correct caloric intake for control animals when the effect on carcinogenesis of metabolic stimulants or depressants is studied. The adequacy of a control group fed at the same gross calorie level is doubtful.

SUMMARY

1. The relationship of dietary fat to the incidence of carcinomas induced in mice with carcinogenic hydrocarbons was investigated. Synthetic type diets were fed in which caloric intake was varied by removing glucose from the ration and the level of fat was increased from 2 per cent to 27 per cent or to 61 per cent by isocaloric substitution for glucose.

2. The diet containing 61 per cent fat and no carbohydrate stimulated carcinogenesis about the same as diets containing 27 per cent fat. This suggested that dietary fat *per se* does not promote the formation of skin carcinomas induced with benzpyrene.

3. When the tumor promoting action of diets containing 2 per cent and 27 per cent fat were compared at two calorie levels it was shown that the greater net energy value of the higher fat diet was sufficient to account for all of the stimulating effect of the 27 per cent fat diet on tumor formation.

4. For a true appraisal of the effect of the three major dietary components on the induction of tumors, the diets should be fed on the basis of an equal net energy value rather than isocaloric allowances calculated from the gross caloric values of the dietary components.

5. Mice that were fed a diet containing 61 per cent of fat and no carbohydrate for 254 days gained weight and appeared normal.

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The Influence of Vitamins of the B Complex on the Induction of Epithelial Tumors in Mice[1]

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Various dietary constituents have been reported to influence the formation and growth of tumors. Because of the essential nature of the vitamins for growth and metabolism, the possible effects of these factors on carcinogenesis have been investigated repeatedly. In addition, there have been searches for specific effects on the cancer process by less clearly defined dietary factors. The reports of many of these investigations have been reviewed by others. (3, 13, 15).

Further investigation designed to clarify the effect of certain dietary factors on tumor genesis appeared appropriate, especially because of recent progress pertinent to the problem. First there have been major advances in the knowledge of the role of B vitamins in nutrition, along with the availability of these compounds in pure, synthetic form. Second, it has been shown that small changes in caloric intake affected tumor formation in mice (2, 19). Thus, any treatment which changed the caloric intake of the animals would affect the tumor incidence. Undoubtedly an uncontrolled caloric intake has invalidated the interpretation of some past investigations.

In the experiments reported here the influence of synthetic diets containing different levels of crystalline B vitamins and of natural diets on the formation of tumors of the skin was studied in mice maintained on isocaloric levels of diet.

EXPERIMENTS AND RESULTS

The materials and general methods of these experiments were described in the preceding paper (2).

Experiment 1.—The rate of tumor formation was studied in 7 groups of 48 female mice fed synthetic diets which varied in their content of 10 synthetic B vitamins. These groups were designated as: 1. control; 2. all vitamins high; 3. all vitamins low; 4. thiamine and riboflavin low; 5. pyridoxine low; 6. niacin and pantothenic acid low; and 7. biotin, pteroylglutamic acid (PGA), p-aminobenzoic acid (PABA), inositol, and choline low. Group 7 was called 5-low for brevity.

A mild degree of restriction was imposed on all groups so that isocaloric feeding would be possible even if the appetite of mice fed one of the low vitamin diets was somewhat decreased. This was done by reducing the amount of glucose in the *ad libitum* diet described previously (2) from 78 to 67.3 parts. The ration then furnished 9 cal. per mouse each day when fed at a level of 2.55 gm. It had the following composition: glucose monohydrate 67.3; crude commercial casein 15; corn oil 2 (reenforced with 6 drops of halibut liver oil per 20 gm. of corn oil, which furnished 21 units of vitamin A and 0.3 units of vitamin D per mouse each day); liver concentrate[2] 1; and salt mixture[3] 4. This diet when supplemented with the amounts of vitamins shown in column 1 of Table 1 became the control diet; with the amounts in column 2 it became the all high vitamin diet; with the amounts in column 3 it became the so-called all vitamins low diet; and so on for the last 4 diets. Crude casein, instead of a vitamin-free product, and liver concentrate were included in these diets to supply possible unknown nutritive factors. Thus, only the influence of controlled variation of known vitamins in a nutritionally acceptable diet was studied. However, it was necessary to omit the liver concentrate from the last 4-named diets in order to achieve the desired low level of certain vitamins. It was replaced with 0.5 part of glucose monohydrate.

The calculated amount of vitamins ingested daily by each mouse in each of the 7 groups is

[1] This investigation was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council. We express our thanks to Dr. Augustus Gibson of Merck and Company for supplies of the B vitamins and to Dr. B. L. Hutchings of the Lederle Laboratories for the pteroylglutamic acid and the pteroyltriglutamic acid.

[2] Liver powder, 1:20 concentrate, for which we are indebted to Dr. David Klein of the Wilson Laboratories, Chicago.

[3] Phillips, P. H., and Hart, E. B. Jour. Biol. Chem., 109: 657, 1935.

shown in Table 2. These calculations include estimates of the amounts contributed by the liver concentrate and the casein, based on analysis of identical material as shown in Table 3. The levels of vitamins in italics in Table 2 were intended to be near the daily requirement of the mouse for maintenance purposes (14). When the minimum requirement of the vitamin was unknown, it was provided at a low yet reasonable level. The goal of the vitamin restriction was to approach as closely as possible a vitamin depletion but at the same time to avoid an acute deficiency which could result in death or in a decreased food intake before the experiment was completed.

TABLE 1

THE AMOUNT OF B VITAMINS ADDED TO THE RATIONS
(MG. PER 15 GM. OF CASEIN)

	Control	All high	All low	Thiamine-riboflavin low	Pyridoxine low	Niacin-panto. low	5 low
Thiamine	0.3	2.0	0.155	0.180	0.3	0.3	0.3
Riboflavin	0.3	3.0	none	0.035	0.3	0.3	0.3
Pyridoxine	0.3	3.0	"	0.3	0.0288	0.3	0.3
Niacinamide	0.5	4.0	"	0.5	0.5	0.24	0.5
Ca pantothenate	2.0	4.0	"	2.0	2.0	0.425	2.0
Biotin	0.01	0.1	"	0.01	0.01	0.01	none
PGA*	0.1	0.5	"	0.1	0.1	0.1	"
PABA†	100.0	100.0	2.0	100.0	100.0	100.0	2.0
Inositol	100.0	100.0	2.0	100.0	100.0	100.0	2.0
Choline	200.0	200.0	2.0	200.0	200.0	200.0	2.0

* PGA: Pteroylglutamic acid

† PABA: p-Aminobenzoic acid

TABLE 2

THE CALCULATED CONTENT OF B VITAMINS IN THE RATIONS*

	Control	All high	All low	Thiamine-riboflavin low	Pyridoxine low	Niacin-panto. low	5 low
Thiamine	9.8	58	5.7	*5.7*	9.1	9.1	9.1
Riboflavin	21.5	98	13.0	*2.85*	10.3	10.3	10.3
Pyridoxine	9.8	87	1.3	8.9	*1.0*	8.9	8.9
Niacinamide	99.0	194	85.0	15.9	15.9	*8.5*	15.9
Ca pantothenate	72.0	129	14.7	59.0	59.0	*14.2*	59.0
Biotin	0.36	3	0.08	0.3	0.3	0.3	*0.03*
PGA	4.0	15	1.2	2.9	2.9	2.9	*0.08*
PABA†	2.8 mg.	2.8 mg.	56.0	2.8 mg.	2.8 mg.	2.8 mg.	56.0
Inositol†	2.8 mg.	2.8 mg.	56.0	2.8 mg.	2.8 mg.	2.8 mg.	56.0
Choline†	5.6 mg.	5.6 mg.	56.0	5.6 mg.	5.6 mg.	5.6 mg.	56.0

* The total amount in μg. present in the daily ration (2.55 gm.) of each mouse.

† The values for PABA, inositol, and choline include only the amounts added to the diet.

After a 5-week period to allow physiological equilibration of the mice to the dietary vitamin level, the first of 35 semiweekly applications of a 0.3 per cent solution of benzpyrene in benzene was given. The experiment was terminated at 178 days and the cumulative tumor counts at that time are given in Table 4. The incidence of carcinomas ranged between 61 and 75 per cent for all groups except that fed the all vitamins low diet, in which the incidence was 39 per cent. The mice fed at the highest vitamin level ingested from about 10 to over 100 times the amounts of the 10 B vitamins as did the all vitamins low mice (Table 2). In spite of this wide range of vitamin intake over a long period of time, the tumor incidence of 6 of the 7 groups was essentially the same.

That the low levels of vitamins were near the critical requirements of the mouse was indicated in two cases. On the thiamine-riboflavin low diet, 6 of the mice showed polyneuritis at 114 days (after 149 days on the diet). The pathological signs disappeared rapidly after a single intraperitoneal injection of 10 μg. of thiamine per mouse. Again at 138 days, 3 more mice were found with polyneuritis and were also given 10 μg. of thiamine. The second observation of acute vitamin deficiency was noted in some of the mice fed the diet low in all B vitamins after 79 days on the diet. The ears of these mice were inflamed and edematous and the paws were scaly and raw. There was considerable loss of hair over the forehead, eyes and nose. The tail was not affected. The resemblance of the condition to rat acrodynia was striking. Because of the condition, the group was fed

the control diet (2.5 gm. per mouse) for one day only on the eighty-third day and the evidence of deficiency disappeared in 3 or 4 days. The same syndrome appeared again 78 days later. It was found that the mice responded to a single dose of 10 μg. of pyridoxine given intraperitoneally, but

TABLE 3

AN ESTIMATE OF THE AMOUNT OF VITAMINS CONTRIBUTED DAILY TO EACH MOUSE BY THE LIVER CONCENTRATE (20) AND BY THE CRUDE CASEIN (4)

	0.426 gm. of casein μg.	0.028 gm. of liver concentrate μg.
Thiamine	0.6	0.7
Riboflavin	1.8	11.2
Pyridoxine	0.35	1.0
Niacin	1.7	84.0
Pantothenic acid	2.2	12.5
Biotin	0.03	0.05
Folic acid	0.08	1.12

not to 50 μg. of calcium pantothenate per mouse each day for 3 days nor to 1 μg. of biotin daily for 3 days, both given intraperitoneally. Thus the dermatitis appeared to be due specifically to a pyridoxine deficiency similar to the acrodynia described by Birch, *et al.* (1) for the rat. In contrast to these observations in the all vitamins low group, mice fed the diet low only in pyridoxine showed no deficiency symptoms, although the calculated pyridoxine intake was lower for the latter group of mice (Table 2). The difference in the health of the two groups may reflect an error in the estimate of the pyridoxine content of the liver and casein samples, or a low availability for the mouse of pyridoxine from the liver and casein.

The 9 calories of ration allotted each mouse daily were eaten throughout the experiment except that traces of food were left during the periods when acute deficiencies were apparent. In general the ration was largely consumed within 5 hours after feeding.

The average weight of each group on the day the mice were placed on the experimental diets is shown in Table 5. Weights are also given at the time of the first application of benzpyrene and shortly after the last application. Beyond 128 days weight records were of less significance because of the rapid development of tumors. The growth response of the 7 groups was quite similar although there was a tendency for the weights to vary directly with the supposed nutritive value of the diets.

Experiment 2.—The second experiment was designed to compare the incidence of tumors in mice fed a highly purified diet with that in another group of mice which received the same control diet that was described in experiment 1. The purified diet differed from the control diet in that the liver concentrate was replaced by 0.5 part of glucose and the crude casein was replaced by alcohol extracted casein.[4] The purified diet was made up fresh each week to lessen the possibility of rancidity.

Two groups of 48 female mice were fed the respective diets at a level of 9.5 cal. per mouse each day (2.7 gm. of each diet, in which the glucose content was increased by 5.7 parts over that of the diets in experiment 1 because of the higher caloric allowance in this experiment). After 18 days the first of 44 semiweekly applications of benzpyrene was given. At 119 days the first carcinoma appeared and the experiment was terminated 217 days after the first application of hydrocarbon. There was no difference in the rate of tumor incidence of the 2 groups (Table 6). The average weights of the mice were also the same. At the time of the first application of carcinogen the average weight of the control group was 22.7 gm. and of the experimental group 23.0 gm.; at 140 days the average weights were 28.3 gm. and 28.7 gm. respectively.

Experiment 3.—The tumor incidence of groups of mice fed 4 different diets was determined. The

TABLE 4

THE INCIDENCE OF TUMORS IN MICE FED RATIONS VARYING IN CONTENT OF B VITAMINS

		Cumulative Tumor Count 178 Days			
		Number of mice			Per cent
Type of ration	Effectual total at 108 days	Alive and tumor free	Papilloma	Carcinoma	Carcinoma
Control	45	3	13	28	62
All high	43	3	4	32	75
All low	46	10	12	18	39
Thiamine-ribo. low	45	4	5	31	69
Pyridoxine low	45	5	7	28	65
Niacin-panto. low	43	4	9	26	61
5 low	43	1	7	32	75

[4] General Biochemicals, Inc., Chagrin Falls, Ohio.

first group was fed a control diet identical to that described in experiment 1 but with glucose at 78 parts. The diet of the second group was composed of ground whole wheat 66, whole milk powder 33, and sodium chloride 1. The diet of the third group was composed of ground polished rice 90, crude casein 3 (plus the amounts of vitamins shown in column 1 of Table 1), corn oil 2 (plus halibut liver oil at the level described in the first experiment), liver 1:20 powder 1, and salt mixture 4. The fourth group was fed a diet similar to the control diet with the exception that PGA was omitted and pteroyltriglutamic acid (PTGA) was added at a level which furnished 8 μg. daily per mouse. This diet was made up weekly to lessen the possible loss of PTGA activity.

TABLE 5

A SUMMARY OF THE WEIGHT RECORD OF MICE FED RATIONS VARYING IN CONTENT OF B VITAMINS

	Number of days on diets*		
	1	35	128
Type of ration	Average weight of mice (gm.)		
Control	28.3	25.9	30.2
All high	27.8	26.0	30.2
All low	28.4	24.6	28.9
Thiamine-ribo. low	27.6	25.0	28.3
Pyridoxine low	27.8	25.2	29.2
Niacin-panto. low	28.7	25.7	30.4
5 low	28.6	25.6	29.1

* The first application of benzpyrene was given on the thirty-fifth day.

The 4 diets were designed for consumption at a level of 10 calories per mouse daily. The control and the PTGA diets contained 78 parts of glucose and furnished 352 cal./100 gm. (2). The whole wheat and whole milk powder diet furnished 408 cal./100 gm. and the rice diet, 353 cal./100 gm.[5]

Groups of 48 female mice were fed the 4 diets for 7 days before the first of 30 semiweekly applications of 0.3 per cent methylcholanthrene in benzene was given. By the forty-eighth day after the first application, the 10 calorie allowance exceeded the actual consumption of the mice on all diets and so rations were cut to 9.5 calories per mouse each day. This was accomplished by a decrease of 5 per cent in the weight of the daily allotment of each of the rations, with no adjustment made in the composition of the diets. The condition of the mice fed the whole wheat-whole milk powder diet appeared unusually good, while the fur of the mice fed the rice diet was rough and thin.

There was no difference in the incidence of carcinomas among the four groups. A summary of the weight records and tumor incidence is given in Table 7.

[5] Caloric values of 500 cal./100 gm. of whole milk powder, 368 cal./100 gm. of whole wheat, and 351 cal./100 gm. of polished rice were assumed in the calculations (5).

DISCUSSION

The relationship between vitamins and tumor induction is generally undefined with the exception of the effect of riboflavin on the development of liver tumors in the rat induced by the ingestion of certain azo dyes (15). Until recent years the fragmentary knowledge of the B-complex prevented systematic approaches to this problem. Furthermore, the experiments of Tannenbaum (19) and others (2) which relate tumor incidence to caloric intake emphasize that the nutritional value of an experimental diet can be an important modifying factor in carcinogenesis because of accompanying changes in caloric intake. Hence in experiments with uncontrolled food consumption, differences in tumor incidence may be a direct result of altered caloric intake rather than a specific effect of the dietary factor involved. The experiments reported here were controlled as to the vitamin level and the caloric intake of the mice.

In these experiments the approach to the prob-

TABLE 6

THE INCIDENCE OF TUMORS IN MICE FED A HIGHLY PURIFIED RATION

			Cumulative Tumor Count					
			Number of mice		Per cent of mice with carcinoma			
Type of ration	No. of mice alive 119 days (effectual total)	No. of mice alive and tumor-free 217 days	Papilloma 217 days	Carcinoma 217 days	133 days	168 days	182 days	217 days
Control	39	4	5	25	8	36	46	64
Purified	39	9	2	26	5	46	54	67

TABLE 7

A SUMMARY OF THE INCIDENCE OF TUMORS AND OF BODY WEIGHT OF MICE IN EXPERIMENT 3

Type of ration	Mean weight of mice in gm. 105 days	No. of mice alive 105 days (effectual total)	Per cent of mice with carcinoma 164 days
Control	28.1	46	83
Whole wheat-milk	30.3	44	77
Rice	27.3	45	87
PTGA	28.2	45	85

lem concerning the influence of nutrition on tumor development followed two distinct courses. The first was an attempt to examine the role of the known B vitamins in the process. A synthetic-type diet was employed and each of the crystalline B vitamins was offered to mice throughout a wide range of levels, the lowest of which were compatible with survival and a reasonable caloric intake throughout the experiment. An attempt was also made to discover whether nutritional factors affecting tumor incidence exist in natural materials. However, the choice of natural dietary factors is tremendous and it is possible to investigate only a limited number. The selection of the whole wheat-whole milk powder mixture as a natural ration was based on the report of Schneider and Webster (16) which demonstrated that the diet was of superior nutritive value for mice and that it contained a resistance factor against certain infections in mice. The rice diet was chosen because polished rice has been reported as a source of tumor inhibitor substances (10).

Since no difference was found in the rate of tumor formation in mice fed either the highly purified, the synthetic diet containing crude casein and a liver concentrate, or the natural diets, a broad distribution of unknown dietary factors which alter the rate of tumor formation is improbable. Hence, the use of synthetic diets in studies on dietary factors in cancer research is sound and is recommended. Such diets allow more accurate control of all dietary components including calories, greater latitude in the variation of the constituent parts, and are more uniformly reproducible.

It is unlikely that high levels of the known B-vitamins exert any inhibiting action on tumor induction since increases of 10 to 100 times over an adequate dietary level of the 10 crystalline B vitamins had no effect on tumor incidence. Furthermore, with the exception of the all vitamins low diet, low levels of the B vitamins which approached the minimal daily requirement of the mouse showed no effect on tumor genesis.

A few comments are necessary when the present negative results with minimal vitamin levels are compared with certain investigations in which the vitamin content has an effect on tumor growth. First, the positive response to a certain vitamin deficiency may be limited to a certain type of tumor or species of animal, and is therefore an effect unique to a specific situation. For example, riboflavin appears to play a role in the metabolism of the carcinogenic azo dyes (7) and thereby tends to protect against liver tumors induced by this agent (12). However, riboflavin does not retard the formation of certain other tumor types (9, 18). Thus, such a result is not a universal one and cannot be presented as evidence for the general anticarcinogenic effect of riboflavin. Furthermore, the degree of vitamin depletion is also an important factor determining tumor response. Little, *et al.* (11) found that diets free of "folic acid" retarded the growth of transplants of the Rous sarcoma in chickens. Such an experiment cannot be readily duplicated in mice since, because of intestinal synthesis, they cannot be depleted of this vitamin as can chickens. It is possible that lower dietary levels of vitamins than those tested in the present experiment might influence the incidence of tumors induced with benzpyrene, but such levels are difficult to achieve in long term experiments without an accompanying decrease in both the caloric intake and the survival rate of the animals. The experiment with the chickens tested the "takes" of tumor transplants and was of short duration. It can definitely be stated from the data presented that wide variation in the dietary level of 10 crystalline B vitamins has no effect on the formation of tumors due to benzpyrene.

The only diet that influenced tumor formation was the one designated all vitamins low. The incidence of tumors in mice fed this diet was approximately half that observed in other groups. This was interpreted as a reflection of the pyridoxine deficiency exhibited by these animals and is supported by inspection of the daily vitamin intake of these mice (Table 2). Kline, *et al.* (8) demonstrated a decreased formation of epithelial tumors in mice fed animal amounts of this vitamin. Stoerk (17) reported that most implants of lymphosarcoma failed to take in mice deprived of pyridoxine, and the administration of a pyridoxine antagonist, desoxypyridoxine, induced regressions in established tumor transplants. However, that the present result was a response peculiar to the combination of vitamins at low levels in the diet cannot be excluded.

In the rat a deficiency of pyridoxine may result in a specific acrodynia (1), but both Morris (14) and Foy and Cerecedo (6) reported failure in attempts to produce this syndrome in mice. In the course of these studies, an acrodynia specific to a pyridoxine deficiency was observed in mice, apparently for the first time.

SUMMARY

The influence of variations in the amount of vitamins of the B complex and of unknown nutritive factors on the formation of tumors induced by

applications of carcinogenic hydrocarbons on the skin of mice was investigated. The food intake was controlled at isocaloric levels within each experiment. The various diets tested can be characterized as follows: 1. control; 2. all vitamins high; 3. all vitamins low; 4. thiamine and riboflavin low; 5. pyridoxine low; 6. niacin and pantothenic acid low; 7. the remaining 5 crystalline B vitamins low; 8. pteroyltriglutamic acid substituted for pteroylglutamic acid; 9. whole wheat-whole milk powder; 10. rice; and 11. a highly purified diet. The rate of tumor induction was not influenced by these various regimens with the exception of diet number 3. This diet resulted in a lower tumor incidence and an acrodynia in the mice which was relieved by the administration of pyridoxine.

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The Binding of Phenolsulfonephthalein by Serum and by Albumin Isolated from Serum in Cancer*

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It is recorded in this paper that the binding capacity of albumin in the serum of cancerous patients for phenolsulfonephthalein (PSP) is often reduced below that of normal individuals and that it is restored in the albumin fraction prepared by the method of Cohn et al. (5) The binding capacity of proteins, a measure of the availability of certain cationic centers in the molecule, and the iodoacetate index are correlated in the present study.

The binding of PSP by serum albumin was first observed by Grollman (10). Since then many cases have been observed where albumin forms complexes with organic and inorganic anions, many with considerable physiologic and pharmacologic significance. Serum albumin is outstanding among native proteins for its binding capacity. Albumin and dye or other anions combine on the basic side of the iso-electric point although each carries a negative charge.

The method of studying anion binding by proteins include: the penetration of dyes added to serum into gelatin (2); attempts at removal of free dye by charcoal adsorption after mixing dye and serum, with estimation of the unadsorbed protein-bound dye (9); electrophoresis (21); changes in the absorption spectrum of dyes caused by association with the protein (18, 24); and equilibrium dialysis (6, 19); the last method was employed in the present study.

There is good evidence that anions are bound to proteins both by electrostatic attachment and by molecular attraction of the van der Waals type. Electrostatic fixation apparently occurs through cationic residues of the basic amino acids (21, 23), of the protein particularly of the ϵ-ammonium group of lysine (18) and less importantly to the guanidinium group of arginine (15, 19). Acetylation of albumin with a consequent decrease of available cationic groups of lysine greatly reduces but does not eliminate the binding of anions (17) suggesting that the guanidinium group of arginine participate to some extent in the binding. Salmine (87 per cent arginine) binds methyl orange (15). Mere possession of these cationic groups seems to be inadequate for anion binding since certain proteins such as β_2 and γ-globulins cannot bind anionic dyes although they contain substantial amounts of lysine and arginine. Guanidination of albumin does not change its affinity for methyl orange indicating that the ϵ-amino group of lysine is not essential as such but only because it furnishes a + charged nucleus (17). As evidence for non-electrostatic molecular attraction is the fact that there is no interaction between anionic dyes and the free amino acids arginine and lysine (15). There is evidence (16) that in binding significance electrostatic attraction outweighs van der Waals action. The relative position of N^+ in the protein structure is a critical factor (17) although the nature of the topology in the protein molecule is still obscure.

In clinical material Bennhold (2) using as a measure the penetration of dyes into gelatin found that the serum albumin of patients with hepatic and renal diseases had decreased binding capacity; these findings were confirmed with the charcoal adsorption method (9). With the latter method Ehrström (7, 8) observed low binding values in 10 patients with tumor cachexia and presented the hypothesis that hypoproteinemia in cancer occurring as a result of increased protein destruction disturbs the synthesis of plasma proteins with the result that proteins with defective adsorptive capacity are fabricated.

A deficiency of -SH groups in the serum in human cancer has been known for some years to occur commonly in cancer based on the following evidence: a) Activation of enzymes. Certain enzymes require free -SH groups for their activity; the activity of dialyzed (inactive) papain (22) or methyl glyoxylase (27) is restored to a greater extent by the addition of blood from normal than from can-

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cerous patients. b) Polarography. The height of the catalytic wave in the polarogram of cobalt, caused by the presence of sulfhydryl compounds, is less with cancer serum than with normal serum (4). c) The reducing power of serum in cancer is frequently decreased (3, 11, 25).

EXPERIMENTAL

Equilibrium dialysis.—Stock solutions containing 100 mg. of PSP were prepared by dissolving the dye in 100 ml. of M/15 phosphate buffer pH 7.4. A calibration curve was made for solutions containing 25 to 100 μgm. of PSP per 100 ml., 1 ml. of 5N NaOH being added just before diluting the solution to 100 ml. The development of color of PSP and its stability has been considered elsewhere (14). An Evelyn photoelectric colorimeter with a 540 mμ filter and matched tubes were used throughout.

Dialysis experiments were done in triplicate. 1 ml. of serum (or weighed protein dissolved in 1 ml. of phosphate buffer) and 1 ml. of stock solution of PSP were placed in a bag of cellulose casing 1.75 cm. ($\frac{2}{3}\frac{2}{2}$ inch) in diameter and 15 cm. long; the bag, knotted at both ends, was placed in a 125 ml. Erlenmeyer flask containing 98 ml. of water and the flask rotated 12 times per minute in a cylindrical rotator for 48 hours at 5° C. After warming to room temperature 10 ml. of the dialysate was diluted to 100 ml. with water with the addition of 1 ml. of 5 N NaOH just before the final volume was reached. Triplicate determinations of the color of the dialysates were made.

To determine the amount of PSP which was bound by the globulin fraction of the serum, one end of the cellulose casing was then cut off and the bag inserted in a graduated 15 ml. centrifuge tube, the closed end of the bag being fastened firmly to the top of the tube by adhesive cellulose tape. By centrifuging for 10 minutes at about 1500 r.p.m. the contents of the bag were transferred quantitatively to the tube where the water-insoluble globulins were thrown to the bottom. The volume of the liquid was measured. The supernatant liquid having been discarded, the precipitate was stirred up in a few ml. of water and again centrifuged. The globulins were dissolved in 0.15 M NaCl and transferred to a 10 ml. volumetric flask, 0.1 ml. of NaOH was added and the color determined colormetrically after filling to the mark.

It was found experimentally that equilibrium between protein solution and dialysate was reached in 40 hours under the conditions described. Although cellulose bags adsorbed larger quantities of PSP, when 1 mg. of PSP was used as in these experiments, less than 10 μgm. was retained by the bag.

A weak Donnan effect operates in the dialysis system; the contents of the bag were 0.18 to 0.36 pH units more acid than the dialysate. The Donnan effect in equilibrium dialysis has been discussed by Klotz and Urquhart (16).

The reproducibility of the results in triplicate determinations was within ±2 per cent. The amount of PSP dye bound by serum albumin was linear with protein concentration in the range 12.5 to 75 mg. (Fig. 1) under the conditions of the experiment. Naturally occurring albumin values in serum greater than 60 mg. per 1 ml. of serum were not encountered in the observations.

Estimations of the albumin content of serum and the iodoacetate index with respect to total protein were made as reported in a previous paper (13).

The results were expressed in forms of a ratio, B/A, expressing the amount of PSP bound per 10 mg. of albumin

$$B/A = \frac{\mu gm.\ of\ PSP\ bound\ by\ 1\ ml.\ of\ serum}{Gm.\ of\ albumin\ per\ 100\ ml.\ of\ serum}$$

Purification of proteins.—Albumin was isolated from approximately 300 ml. quantities of 6 samples of normal serum and 3 sera of patients with cancer by the method of Cohn et al. (5). The albumins obtained all showed a single sharp electrophoretic peak with mobility of 6.37 ± 0.15, characteristic of albumins; no traces of other protein fractions were evident[1]. Micro-analyses for carbon, hydrogen, nitrogen, and sulfur were made on the various albumin fractions. Aliquots of albumin[2] were dissolved in phosphate buffer and the following items were determined: PSP binding; iodoacetate index; amino groups by the method of Van Slyke (26); -SH groups[3] by amperometric titration (1, 20). Similar tests had been made on these sera before isolation of the albumins.

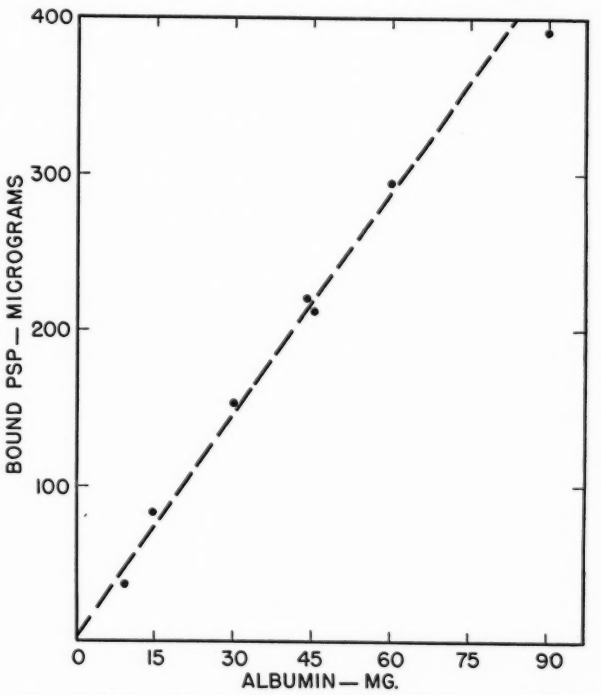

FIG. 1.—Linearity in binding of PSP by serum with an albumin content between 12.5 and 60 mg.

[1] Electrophoretic analysis was carried out by Mr. Bernard Udin, Department of Biochemistry, the University of Chicago.

[2] Microanalyses of purified albumins were carried out by Dr. J. F. Alicino.

[3] We are indebted to Dr. S. G. Weissman for the sulfhydryl determinations.

RESULTS

PSP binding by globulins.—50 mg. lots of plasma proteins[2] prepared by Method 6 of Cohn et al. (5) were tested for PSP binding. The approximate composition of the fractions and the binding values follow: Fraction V, 95 per cent albumin, bound 300 μgm.; Fraction IV-4, 16 per cent albumin and 84 per cent globulins, bound 70 μgm. No PSP was bound by any of the following fractions: II + III, 4 per cent albumin and 91 per cent globulins; IV-1, 100 per cent α-, β- and γ-globulins; a fraction containing over 95 per cent γ-globulin.

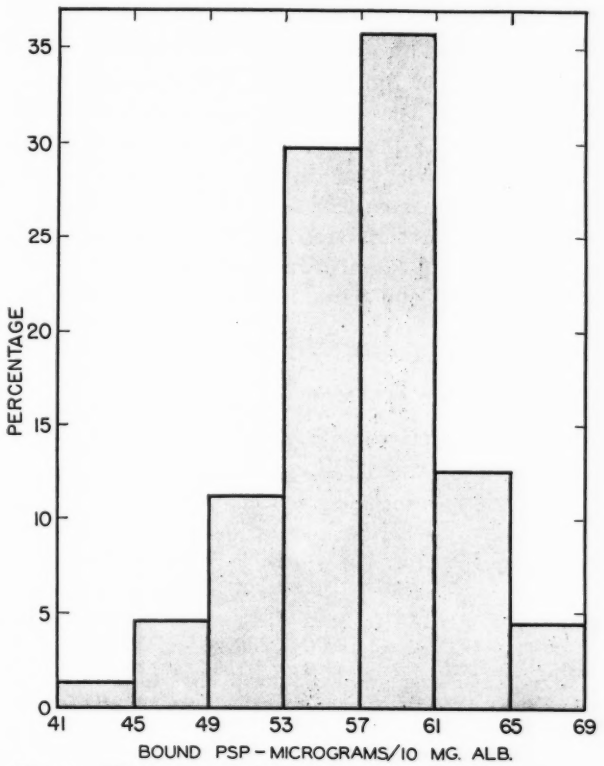

FIG. 2.—Histogram of PSP binding ratio (B/A) of the serum of 156 normal human adults.

The water insoluble globulins of serum bound no dye in any case except in certain myelomas. In 2 cases of multiple myeloma a glassy precipitate, very red with bound dye, was observed; the binding of PSP was 16 and 22 μgm. per 10 mg. of protein respectively. On electrophoresis of these sera the abnormal component migrated slightly slower than or with the γ-globulin peak. In 8 other multiple myeloma sera this binding globulin was not observed.

PSP binding by human serum.—The serum was examined for PSP binding in 418 cases distributed in 3 categories: 156 normal adults; 139 persons with non-malignant disease; 123 persons with cancer. The range of dye bound in the whole series of sera examined varied from 10 to 345 μgm. per 1 ml. of serum.

Relating the bound PSP to 10 mg. of albumin (B/A ratio), the sera of normals (Fig. 2) had higher mean and median values (Table 1) and a smaller standard deviation than serum from cancer patients. Only 2 persons believed to be healthy had a B/A ratio less than 45; the value 45 was accordingly selected as an arbitrary lower limit for the binding of normal human serum.

In the entire series, 81 persons (19.4 per cent) had B/A ratios less than 45. These comprised: 2 in the normal group; 55 cancer patients; 24 persons with diseases other than cancer. The non-malignant diseases associated with these lower values comprised: liver disease, 9; infection, 6; uremia, 3; rheumatoid arthritis, 3; other conditions, 3.

The sera from 123 cancer patients had a median B/A ratio of 46.4. Fifty-five (44.7 per cent) of the patients with cancer had ratios less than 45. While the neoplasms were in an advanced stage in most of the cancer series, some cases of metastatic cancer were encountered in which all of the properties of the serum albumin including PSP binding were normal.

TABLE 1

STATISTICAL ANALYSIS OF PSP BINDING OF SERUM

PSP index: Bound PSP in μgm / Gm. albumin in 100 ml.	No. of patients in series	Range	Median	Mean	Standard deviation
(1) Normals	156	41.0–68.9	57.4	57.07	5.79
(2) Cancer	123	2.8–65.6	46.4	44.61	11.91

The difference between the serum of normal and cancerous persons is more apparent when the binding-albumin ratio of 50 is taken as a critical level: 15 members of the group of 156 normals (9.6 per cent) had B/A ratios of 50 or less while 88 patients in the group of 123 cancerous individuals (71.5 per cent) were in that range.

Correlation of PSP binding with the iodoacetate index.—The iodoacetate index (13) and PSP binding were determined simultaneously on the sera of

[4] We are indebted to Dr. John T. Edsall, Department of Physical Chemistry, Medical School of Harvard University for the gift of some of these fractions.

47 cancer patients. In all of the cancer sera when the B/A ratio was less than 45, the iodoacetate index was also in the pathologic range; whenever the B/A ratio was larger than 50, the iodoacetate index was also normal. There were 12 cases where the B/A ratio ranged from 45 to 50; 9 of these sera had iodoacetate values in the pathologic range and 3 of them in the normal distribution.

Effect of surgical operation on PSP binding and the iodoacetate index.—Both the binding of PSP and the iodoacetate index often decrease markedly in persons with normal original values, subjected to trauma. These phenomena have been most frequently observed in post-operative states and they occur frequently even when a major surgical operation has been carried out with little blood loss and with minimal manipulation of the tissues. Marked lowering has occurred without decreases of total protein or albumin content of the serum and within 8 hours after the operation; the rapidity of the decrease is significant.

Dialysis and fractionation of albumin from normal and cancer serum.—Sera with low PSP binding values from patients with cancer were dialyzed for 2 days against water or 0.15 M NaCl; PSP was then added to the cellulose bag and the binding capacity of the proteins determined in the usual way. No differences were observed on dialysis with water or sodium chloride solutions; in both cases dialysis caused a slight improvement in the binding capacity of the proteins, increases of the order of 3 to 5 per cent being observed, but these values were considered insignificant.

The values given in Table 2 indicate that the relatively large differences in the iodoacetate index of thermal coagulability and in dye binding which exist between the proteins of normal and cancer sera are not in evidence in the albumin fraction purified by the cold ethanol fractionation technique (5). Moreover there are no significant differences in the composition of the albumins isolated from the 2 types of sera as indicated by their content of carbon, nitrogen, hydrogen, and sulfur as well as free amino and -SH groups.

TABLE 2

COMPARISON OF SERUM PROTEINS WITH ISOLATED ALBUMINS

	Original Serum				Isolated Albumin					
Serum Source	Iodoacetate index	Dye binding μgm/10 mg. albumin	Iodoacetate index	Dye binding μgm/10 mg. albumin	SH μm/g	NH_2 μm/g	%C	%H	%N	%S
Normal										
P-2				69.8	11.2	950	51.8	6.98	16.3	1.91
P-3			19.4	71.0	11.4	986	52.9	6.93	15.9	1.85
P-4			19.6	71.7	9.5	921	52.6	7.02	15.9	1.81
H-5	10.9	58.2	18.6	73.2	10.4	928	53.7	7.58	15.7	2.29
J-6	11.1	52.0	17.5	68.8	11.5	878	52.8	6.96	15.8	2.09
W-7	9.8	63.0	18.4	69.5	11.2	907	53.0	7.02	16.1	1.88
Cancer										
P-1	6.0	25.0	15.6	49.5	7.9	1157	53.1	6.97	15.8	2.11
W-2	6.5	26.0	18.6	72.3	10.1	1035	52.8	6.97	16.1	1.85
W-3	6.5	38.2	16.8	66.6	10.0	914	52.6	6.98	16.2	1.94

DISCUSSION

Except with myeloma serum and protein fractions containing globulins all of the values reported in this paper are related to weight of albumin.

In this series an abnormally low capacity (B/A less than 45) for binding PSP was encountered in 44.7 per cent of the sera from cancer patients compared with 17.3 per cent of the sera of patients with a wide variety of serious non-cancerous diseases; all of these cancer sera had low iodoacetate indices. On fractionation of the serum albumin from normal and cancerous persons these defects are eliminated; the refined albumin fractions from both normal and cancer patients resemble one another in the properties examined.

According to current theory the iodoacetate index is dependent on the availability of thiol and certain amino groups (12) in the protein molecule while the binding of dye anions is related to cationic centres with specific, but as yet ill-defined, configurational relationships. The same sera in general show similar defects in both coagulation and dye-binding properties; refinement of the albumin by the cold ethanol procedure largely eliminates both defects. It would appear that the defects are not intrinsic in the albumin molecule, and it is postulated that they arise because of associated groups, possibly of anionic type which prevent the normal reaction of protein groups. To explain the results obtained the binding must be of such a nature that the bound substance is not removed by dialysis but is removed by the cold 40 per cent ethanol of the fractionation procedure.

The speed with which normally reactive albumin acquires defects in PSP binding and thermal

coagulation after a surgical operation is evidence supporting a superficial rather than an intrinsic change in the albumin molecule.

CONCLUSIONS

By equilibrium dialysis it was demonstrated that there was defective binding of anionic dyes in the serum of 44.7 per cent of patients with cancer in this series. Dialysis against water or sodium chloride caused only an insignificant improvement in binding. On refinement of serum albumin from cancer patients by cold ethanol fractionation this defect and also deficiency of thermal coagulation were eliminated. No significant differences were found on elementary analysis or on determination of thiol or amino groups between fractionated normal or cancerous serum albumin.

A marked defect in the binding of PSP and a decrease of the iodoacetate index of thermal coagulability of serum can occur within 8 hours after a surgical operation.

Globulins in refined plasma protein fractions did not bind PSP. The water insoluble globulins of serum likewise failed to bind PSP except in 2 cases of multiple myeloma where a gelatinous precipitate of water insoluble globulins occurred following dialysis; these binding globulins migrated electrophoretically with γ-globulin.

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INTERNATIONAL CANCER RESEARCH COMMISSION MEETING IN PARIS, JULY 18 TO 22, 1949

History.—The Commission was established at the Fourth International Cancer Research Congress held in St. Louis, September 2 to 7, 1947. The American Association for Cancer Research organized this Congress, with the approval of the Department of State and with the cooperation of the Union Internationale Contre le Cancer. Dr. E. V. Cowdry was selected as President of the Congress and appointed appropriate committees to expedite arrangements and finance of the Congress. Invitations to send delegates to the Congress were transmitted by the Department of State to all nations having diplomatic relations with the United States. President Truman appointed the following U.S. delegates:

John J. Bittner
James P. Cooney
E. V. Cowdry
W. U. Gardner
Elise L'Esperance
C. P. Rhoads
Leonard A. Scheele
Shields Warren
Richard B. Williams

Encouraged by the President's letter and telegram to the Congress, recognizing the importance of international cooperation in cancer research and expressing the hope that the cooperation therein achieved "will carry over into other problems of world concern," and with the deepest feelings of urgency, these delegates together with those of 39 other nations recommended to the Congress establishment of an organization to be known as "The International Cancer Research Commission" to operate in accordance with the following principles:

1. That cancer research include all efforts to advance our knowledge of cancer by clinical, experimental, or other means.
2. That the Commission consist of one member from each country here represented, with equal voting power. Other nations not here represented will be welcome on the same basis.
3. That the principal source of financial support should be governmental.
4. That the governments should be invited each to send one national representative who should be actively engaged in cancer research, to serve for a period of three years, and advises that such representation be changed at the end of this three years. Further, that the governments after two years select an additional representative who will serve without vote prior to succeeding the voting nominee.
5. That the Commission should arrange meetings once a year, and never consecutively in the same country.
6. That an executive committee be constituted made up of five members and not more than seven as follows with alternates: one each from Latin America, Asia, United States and two from Europe.
7. That the first executive committee be constituted as follows:

Latin America—Dr. Ignacio Millan (Chairman)
Dr. Felix E. Leborgne, alternate
United States—Dr. E. V. Cowdry
Dr. W. U. Gardner, alternate
Asia—Dr. V. R. Khanolkar
Dr. Tu-Shan Yung, alternate
Europe—Dr. J. Maisin
Dr. A. Lacassagne, alternate
Dr. A. Haddow
Dr. J. Engelbreth-Holm, alternate

8. That the Commission form part of the Union Internationale, replacing the Comité Scientifique. It advises that this Commission, within the organization of the Union Internationale, be given the greatest possible freedom of action to attain its goal, and that it have direct power to solicit and distribute financial aid, which has been agreed to by the Union.
9. That this Fourth International Cancer Research Congress request of governments that the group of national representatives here assembled and its executive committee be permitted to serve as the first appointees.

Official French and Spanish translations of these "principles" were also made and the Congress enthusiastically and unanimously approved.

The forty nations and their representatives, which constitute the charter members of the Commission, are:

Argentina—J. E. Pessano
Australia—R. Kaye-Scott
Belgium—J. Maisin
Bolivia—H. F. Ferrufino
Brazil—A. Prudente
Canada—G. E. Richards
Chile—A. Rahausen
China—T. S. Jung
Colombia—R. A. Garcia
Czechoslovakia—H. Sikl
Denmark—J. Engelbreth-Holm
Egypt—J. N. Azzouni
France—A. Lacassagne
Great Britain—A. Haddow
Greece—E. G. Minopoulos
India—V. R. Khanolkar
Iran—C. Oberling
Iraq—S. Faik
Israel—A. Hochmann
Korea—I. S. Yun
Luxembourg—S. Hertz
Mexico—I. Millan
Netherlands—R. Korteweg
Nicaragua—F. V. Paiz
Norway—L. Kreyberg
Panama—E. Zubieta
Peru—E. Caceras
Philippines—J. A. Arcella
Portugal—M. Pinto
Salvador—D. R. Posada
Siam—C. Ruangsiri
Sweden—E. Ask-Upmark
Switzerland—H. R. Schinz
Tunisia—C. W. Anderson
Turkey—P. Cambel
Union of South Africa—M. Weinbren
United States—E. V. Cowdry
Uruguay—F. Lebrogne
Venezuela—H. Rivero

Finally the U.S. Department of State transmitted to all nations a memorandum on the formation of the Commission.

Attendance at the Paris Meeting.—

Arabia—R. Pharaon
Australia—Douglas Pearce
Austria—J. Kretz
Belgium—J. Maisin
Brazil—A. Prudente
Cuba—N. Puente Duany
Denmark—J. Engelbreth-Holm
Egypt—M. Ashour
Finland—Otto Jarvi
France—A. Lacassagne
Great Britain—A. Haddow
Greece—E. G. Minopoulos
Iceland—N. Dungal
India—V. R. Khanolkar
Iran—A. H. Radji
Israel—A. Hochmann
Jugoslavia—X. Chahowitch
Korea—I. S. Yun
Lebanon—J. Zahar
Luxembourg—S. Hertz
Mexico—I. Millan
Netherlands—R. Korteweg
Norway—L. Kreyberg
Poland—Fr. Lukaszczyk
Portugal—L. Basto
Sweden—E. Berven
Switzerland—H. Schinz
Tunisia—C. Anderson
Turkey—M. Cincin
United States—E. V. Cowdry

Officially accepted as members, but not present:

Bulgaria—R. Papaskov
Indochina—M. Long

Also attending the meeting were additional representatives of many nations; from the United States were W. U. Gardner, alternate member of the Executive Committee, and R. G. Meader representing the U.S. Public Health Service and the American Cancer Society.

Progress Reports.—

1. The Chairman, Ignacio Millan (Mexico) recalled the fact that at the Fourth Congress in St. Louis the Commission was empowered to recommend to the Union Internationale Contre le Cancer the location of the Fifth Congress. In accordance with this directive Paris was recommended as the site and Dr. A. Lacassagne as President.

2. Dr. J. Maisin (Belgium) reported that the Union, and consequently the Commission as a part of the Union, has been incorporated in both WHO and the International Council for the Coordination of International Congresses in Medical Sciences (a UNESCO and WHO subsidiary). He mentioned, among the advantages of this relationship, a grant from UNESCO to help defray the cost of publication of the Transactions of the Fourth Congress. He explained that this publication was almost completed, 4 of 6 parts having already appeared in ACTA; but that the number of subscriptions was very disappointing. The discussion which followed showed that the publication of the Transactions had not been sufficiently advertised, that some individuals and libraries were ignorant of the size (about 2000 pages), of the cost ($25.00) and of the mechanism of ordering both the Transactions and Reprints through Dr. J. Maisin, 61 Voer des Capucins, Louvain, Belgium.

3. Attention was called by E. V. Cowdry (United States) to the progress that has been made in expanding the Commission by the addition of 11 new member nations to the 40 charter members, making a total of 51. The following have officially expressed interest in the Commission, which it is hoped will develop into membership and active participation:

Afghanistan	Honduras
Dominican Republic	Costa Rica
Eire	Paraguay
Ecuador	Liberia
Ethiopia	Java (Indonesia)
Hungary	U. S. S. R.

The goal set for 1950 is all nations.

4. The national representatives presented reports on advances in cancer research in their several countries which it is not feasible here to summarize. Much interest was shown in mimeographed summaries of expenditures by the American Cancer Society and other organizations prepared by J. H. Teeter. The Texas and Michigan Cancer Bulletins were exhibited. Dr. Ralph Meader of the U.S.P.H.S. reported briefly on governmental financing of cancer research. Dr. W. U. Gardner spoke about the publication of cancer atlases and reviews of cancer literature—a service repeatedly urged by the national representatives. It was unanimously agreed that such annual progress reports are of great value and that more time should be devoted to their discussion.

New Business.—

After long discussion by the Executive Committee of the Commission it was unanimously agreed that:

The duties of the International Cancer Research Commission are to advance research on cancer:

1. By serving as the Conseil Scientifique of the Union.
2. By pooling information at annual meetings and on specific topics at special small meetings.
3. By obtaining more agreement on cancer nomenclature through the publication of atlases and other means.
4. By giving advice in the planning of cancer research when asked.
5. By helping to secure equipment and supplies when asked.
6. By promoting studies on environmental (occupational and geographic) pathology of cancer.
7. By facilitating adequate review of the cancer literature.

Serving as Conseil Scientifique to the Union Internationale Contre le Cancer involves mainly giving advice regarding the research programs of the large International Cancer Congress held every three years.

Because progress is now being made in the direction of agreement on cancer nomenclature by the publication of atlases and by other means on both sides of the Atlantic, which it is important to bring together, a committee was appointed to carry on consisting of *Perry*, Millan, Denoix, and Meader.

The geographic pathology of cancer is a problem in which the Commission is obviously qualified to take a leading part. This duty was assigned to a committee made up of *Millan*, Khanolkar, Maisin and Engelbreth-Holm.

In order to profit by an excellent beginning made by

Meader in the registry of cancer research institutions, equipment and workers, it was decided that Meader would supply Millan with copies of a suitable questionnaire which Millan would forward to all members of the Commission with the request that it be answered and returned direct to Meader.

The question of an adequate review of cancer literature was left to *Khanolkar*, Korteweg and Lacassagne acting as a committee to make tentative plans, to send these plans to all members of the Commission for suggestions and to submit final plans to the Executive Committee of the Commission.

These duties of the Commission, as defined by the Executive Committee, were presented in English and in French to the entire Commission and approved. They now are incorporated into the charter of the Commission with the principles adopted by the Fourth Congress in St. Louis.

The location of the next annual meeting of the Commission was discussed. Item 5 in the "Principles" of organization provides that annual meetings shall never be held consecutively in the same country. To have the 1950 meeting in Paris, where the 1949 meeting was then in session, would be inconsistent with this principle; but it would be convenient because all members of the Commission would be in Paris attending the Fifth Congress. To accept a cordial invitation from Portugal to have the 1950 meeting in Lisbon would necessitate much additional travel and expense. In deciding in favor of the first alternative of having the 1950 meeting in Paris, all members of the Commission affirmed their belief in the principle of change in location and agreed that this action did not constitute a precedent. The meeting will therefore take place immediately before the V Congress (July 15–22, 1950) in Paris where the entire membership can be accommodated in a single hotel to be selected. Detailed arrangements were left to Dr. A. Lacassagne, representative of France on the Commission. It is expected that official invitations will be dispatched by the French Foreign Office as was the procedure in the case of the present meeting.

Members.—In the following list of members the number of years each individual will have served at the 1950 meeting is given after the name. The Commission is committed to the principle of a change in membership after 3 years or earlier in order to spread the responsibility and to obtain the support of a steadily increasing group of people familiar through experience with the working of the Commission. However, governments are of course free to delegate whom they wish for as long as they desire to serve on the Commission. Probably most of the third year class will be replaced without difficulty.

MEMBERS OF INTERNATIONAL CANCER RESEARCH COMMISSION
JULY 22, 1949

ARABIA: R. Pharaon, Minister of Arabia, Paris (1st year)

ARGENTINA: Juan Esteban Pessano, Secretaria De Salud Publica de la Nacion Direccion de Neoplasias, Pozos 2073, Buenos Aires (3rd year)

AUSTRALIA: Douglas Pearce, 12 Collins Street, Melbourne (1st year)

AUSTRIA: J. Kretz, Brahmsplatz 5, Vienna IV (1st year)

BELGIUM: J. Maisin, University of Louvain, 61, Voer des Capucins Louvain (3rd year)

BOLIVIA: Hector Fernandez Ferrufino, Hnos, Machego No. 908, La Paz (3rd year)

BRAZIL: Antonio Prudente, Benjamin Constant 171, Sao Paulo (3rd year)

BULGARIA: R. Papasov, Rue Strandja 136, Sofia 2 (1st year)

CANADA: G. E. Richards, Ontario Cancer Treatment & Research Foundation, Toronto (Deceased, awaiting new representative)

CHILE: Alberto Rahausen, Chief of Curietherapy Instituto Nacional del Radium, Avenida Zanartu 1000, Santiago (3rd year)

CHINA: Tu-Shan Jung, Dept. of Radiology, Peking Union Medical College, Peking (3rd year)

COLOMBIA: Ruben A. Garcia, Instituto del Radium, Bogota (3rd year)

CUBA: Nicolas Puente Duany, K. 503, Vedado, Habana (1st year)

CZECHOSLOVAKIA: H. Sikl, Dept. of Pathology, University Charles IV, Prague (3rd year)

DENMARK: J. Engelbreth-Holm, Universitetets Patologisk-Anatomiske Institut, Frederik V.s Vej 11, Copenhagen (3rd year)

DUCHY OF LUXEMBOURG: Simon Hertz, 7 Grand 'rue et rue du Fosse, Luxembourg (3rd year)

EGYPT: Maamoun A. Ashour, Pathology Section, Public Health Laboratory, Cairo (1st year)

EL SALVADOR: Don Ricardo Posada, Universidad de el Salvador, San Salvador (3rd year)

FINLAND: Osmo Jarvi, University of Turku, Kristünak 1, Turku (1st year)

FRANCE: A. Lacassagne, Laboratoire de Biologie, Institut du Radium de l'Universite de Paris, Rue d'Ulm 26, Paris (3rd year)

GREAT BRITAIN: Alexander Haddow, Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, London SW 3 (3rd year)

GREECE: Efstathios G. Minopoulos, 38 Zaimi Str. Athens (3rd year) (33 Av. Fr. Roosevelt, Avon-Fontainebleau, Paris, France)

ICELAND: Prof. Niels Dungal, University of Reykjavik, Sudurgata 12, Reykjavik, (1st year)

INDIA: Vasant R. Khanolkar, Director of Laboratories, Tata Memorial Hospital, Bombay (3rd year)

INDOCHINA: M. Long, Institut du Cancer, Hanoi (1st year)

IRAN: A. A. Radji, Bankemelli Hospital, Teheran (1st year)

IRAQ: Salman Faik, Assoc. Prof. Royal College of Medicine, Bagdad (3rd year)

ISRAEL: A. Hochmann, Radium and Tumour Institute, Hospital Hadassah, Jerusalem (3rd year)

ITALY: Francesco Pentimalli, Instituto Pathologia Generale, San Andrea Dame 8, Naples (3rd year)

JAPAN: R. Kinosita, Osaka University, Osaka (1st year)

JUGOSLAVIA: X. Chachovitch, University of Belgrade, Belgrade (1st year)

KOREA: Il Sun Yun, Dept. of Pathology, Seoul University Medical School, Seoul (3rd year)

LEBANON: J. Zahar, 36 Avenue d'Italie, Paris, 13 (1st year)

MEXICO: Ignacio Millan, Director of Tumor Clinic, General Hospital, Avenida Veracruz 69, Mexico, D.F. (3rd year)

NETHERLANDS: R. Korteweg, Laboratorium Antoni Van Leeuwenhoek-Ruis, Sarphatistraat 108, Amsterdam (3rd year)

NEW ZEALAND: Sir James Elliott, % 43 Kent Wellington, New Zealand (1st year)

NICARAGUA: Fernando Velez Paiz, Managua, D.N. (3rd year)

NORWAY: Leiv Kreyberg, Dept. of Pathology, University of Oslo, Oslo (3rd year)

PANAMA: Ernesto Zubieta, P.O. Box 1595, Panama City (3rd year)

PERU: Eduardo Caceres, Dept. of Anatomy, San Marcos University, Lima (3rd year) at present—Memorial Hospital, 444 East 68th Street, New York City, N.Y.

PHILIPPINE REPUBLIC: Juan A. Arcellana, College of Medicine & Philippine General Hospital, University of Philippines, Manila (3rd year)

POLAND: Fr. Lukaszczyk, Director, Institute of Radium, 15 Rue Wawelska, Warsaw (1st year)

PORTUGAL: Lima Basto, 21 Av. Defensores Chaves, Lisbon (1st year)

SIAM: Chanai Ruangsiri, Chulalankarana University, Bangkok (3rd year)

SWEDEN: Elis Berven, Clinical Director of the Radiumhemmet, Caroline Hospital, Stockholm (1st year)

SWITZERLAND: H. R. Schinz, Röntgeninstitut & Radiotherapeutische Klinik, Kantonsspital, Zurich (3rd year)

REGENCY OF TUNISIA: Charles W. Anderson, Directeur du Laboratoire de Medicine Experimentale de l'Institut des Hautes Etudes de la Regency, Tunis (3rd year)

TURKEY: Macit Cincin, 28 Place Denfert-Rochereau, Paris (1st year)

UNION OF SOUTH AFRICA: Lt. Col. Maurice Weinbren, X-ray Dept., Chamber of Mines Hospital, P.O. Box 774, Johannesburg (3rd year)

UNITED STATES OF AMERICA: E. V. Cowdry, Prof. of Anatomy, Washington University Medical School, St. Louis, Mo. (3rd year)

URUGUAY: Felix Leborgne, Ibicuy 1210, Montevideo (3rd year)

VENEZUELA: Hermogenes Rivero, Instituto de Oncologia, Luis Rozetti, Caracas (3rd year)

Signed E. V. COWDRY
W. U. GARDNER
Delegates of the American Association for Cancer Research

New Books

The Basis of Chemotherapy. By THOMAS S. WORK, B.Sc., Ph.D.; Research Staff, National Institute for Medical Research, London, and ELIZABETH WORK, B.A., PH.D.; Research Staff, Department of Chemical Pathology, University College Hospital Medical School, London. New York, N.Y.: Interscience Publishers, Inc., 1948, Pp. 435. $6.50.

Chemotherapy touches on many sciences including organic chemistry, biochemistry, physical chemistry, bacteriology, pharmacology, and therapeutics. Although the term is here restricted to the chemical treatment of diseases of proved microbial origin, there is much of value for tumor chemotherapy. A brief but interesting historical introduction traces the subject prior and through Ehrlich's work to the sulfa drugs and antibiotics. In a chapter on cell metabolism the functions of enzymes in intermediary metabolism are discussed with the possibility in mind of explaining drug action on the basis of interference with enzymes. A chapter on essential metabolites leads to metabolic analogues as growth inhibitors, and to chapters on enzyme inhibition, drug antagonism, drug resistance, and the relation of chemical structure and activity.

This book is so clear and packed with information that the reviewer found it highly stimulating. Its timely appearance should make it welcome to workers interested in cancer metabolism and therapy.

Advances in Enzymology and Related Subjects in Biochemistry: Volume 9. Edited by F. F. Nord. New York, and London: Interscience Publishers, Inc., 1949. Pp. 760. $9.00.

This volume resembles its predecessors, with which most of our readers are no doubt familiar, in having a high standard of interest and value. The different chapters cover a wide range of subjects, some of which should be of direct concern to workers with enzymes in cancer.

Physiology in Diseases of the Heart and Lungs. By M. D. ALTSCHULE; Assistant Professor of Medicine, Harvard Medical School, Visiting Physician and Research Associate, Beth Israel Hospital; Director of Internal Medicine and of Research in Clinical Physiology, McLean Hospital. Cambridge, Mass.: Harvard University Press, 1949. Pp. 368. $5.00.

This book is number 10 in the Harvard University Monograph in Medicine and Public Health series. It is written to help medical students with inquiring minds bridge gaps between the basic sciences and clinical instruction. This objective appears to be admirably met. The available information on pathologic physiology in chronic cardiac decompensation, pulmonary edema, cardiac arrhythmias and other conditions is critically evaluated. Extensive bibliographies are printed with each section. Students are fortunate that this scholarly and laudable volume has been published.

Advances in Surgery: Volume I. Edited by William DeW. Andrus. Chairman, Editorial Board. New York and London: Interscience Publishers, Inc., 1949, Pp. 554. $11.00.

Twelve authors have contributed seven sections to this first volume in a new "Advance" series. The subjects include traumatic shock, strictures of the common duct, regeneration of nerves, antibiotics, immersion foot, blood vessel anastomosis, and tumors of bone. Most of the sections present more than recent advances. They cover the respective subjects rather thoroughly, and some include original data. The only section on tumors is that on bone, by Drs. B. L. Coley and N. L. Higinbotham. It is a good clinical discussion of this problem, but it contains what appears to be a greatly over-simplified classification of primary malignant bone tumors.

Biological Applications of Tracer Elements. Cold Spring Harbor Symposia on Quantitative Biology, Volume 13. Cold Spring Harbor, New York: Biological Laboratory, 1948, Pp. 222 plus XI, and 87 figures. $7.00.

The twenty-five papers which comprise this new volume cover a wide range of subjects. They have in common the use of tracer elements. The high standard set by previous symposia is maintained. It is apparent that the high hopes for aid from the biological applications of tracer elements are being realized. Cancer investigators working on chemical problems should have access to this book.

Haemoglobin. Edited by F. J. W. Roughton and J. C. Kendrew. New York and London: Interscience Publishers, Inc., 1949. Pp. 317 and XII. $8.50.

This symposium is based on a conference held at Cambridge in June 1948 in memory of Sir Joseph Barcroft. The subject is thoroughly and capably covered in about 28 sections by his colleagues, friends, and students. This is a scholarly memorial befitting the memory of a great scientist.

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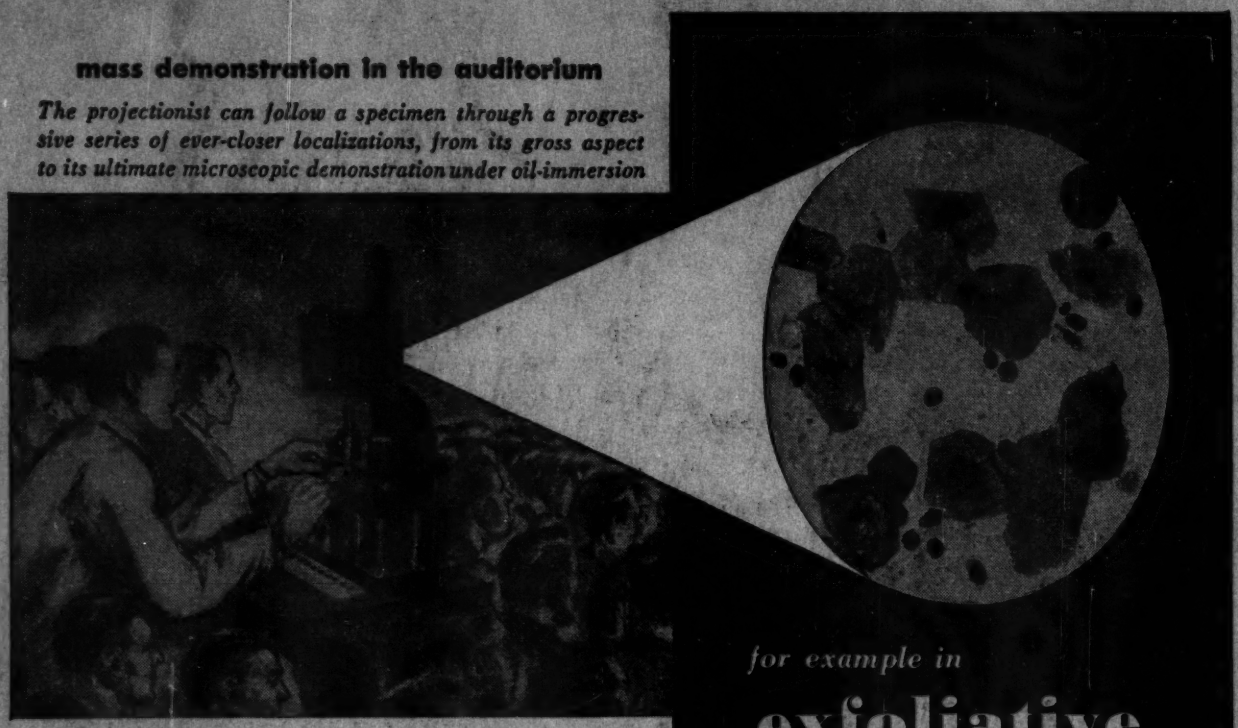

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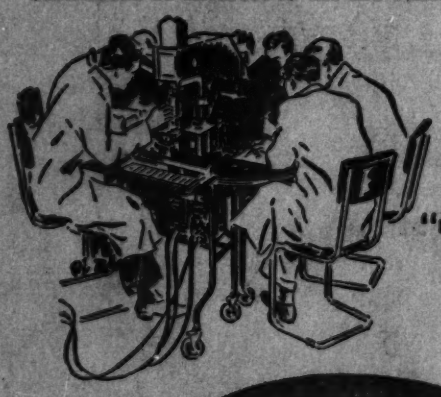

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